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*CENTIMETER WAVELENGTH RADIO ASTRONOMY INCLUDING OBSERVATIONS USING THE MASER**

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During the past fifteen years, radio astronomy observations have been largely confined to radiation at wavelengths longer than about 20 cm. The purpose of this talk is to give a brief outline of the work to date at shorter wavelengths, in particular wavelengths less than 10 cm, and to describe some of the new techniques being used in this work. Since the extensive field of solar radio astronomy has already been reviewed by Dr. Maxwell,* this talk will be concerned only with sources other than the sun.

The radio astronomer who works at cm wavelengths faces a number of difficulties not found at longer wavelengths. He is first of all limited to observing continuum radiation since none of the spectral lines in this region has so far been detected. Secondly, the flux density and hence, for a given antenna, the antenna temperature available from the discrete nonthermal sources are approximately proportional to λ. A third difficulty is inadequate receiver sensitivity. At wavelengths of 1 m and longer receivers have been available with internal noise temperatures lower than a few hundred degrees Kelvin, which is less than the background noise at the galactic pole. At meter wavelengths, improved receiver sensitivity is not of great importance. In the cm region, however, where the background temperature is only a few degrees Kelvin, the crystal noise in superheterodyne receivers has led to receiver noise temperatures of 1000°K or more. At wavelengths shorter than 3 cm crystal noise temperatures as well as atmospheric absorption rise rapidly. Indeed apart from the sun, the moon, and Venus, no discrete sources have been detected at wavelengths between 3 cm and the optical infrared region.

The necessity of large high-precision antennas has also restricted progress at centimeter wavelengths. For example, with the use of standard superheterodyne techniques and a five second averaging time, a parabolic reflector 10 ft in diameter or its equivalent is required merely to *detect* Cassiopeia A at 3 cm wavelength. Clearly, much larger antennas, constructed with surface tolerances of the order of $^1/_4$ inch, are required for research at these wavelengths. Prior to 1958 the only large antenna suitable for observations of discrete sources at cm wavelengths was the 50-ft reflector[1] at the U. S. Naval Research Laboratory.

In spite of the experimental difficulties a number of significant observations have been made, many of them yielding information accessible only at cm wave length.

SUMMARY OF CENTIMETER WAVELENGTH OBSERVATIONS

Spectra of the Discrete Sources.—The initial effort at observing galactic noise at cm wavelength was probably that of Reber in 1938.[2] Although failing to detect 9 cm radiation with relatively insensitive equipment, he was able to conclude that the spectrum of the radiation is far from that of a black body. Working at 10 cm in 1951, Piddington and Minnett[3] with the use of a "beam swinging" technique found an excess flux density of $7 \times 10^{-21}\ w\ m^{-2}\ (c/s)^{-1}$ associated with the region near the galactic center.

Discrete sources were first detected at wavelengths shorter than 21 cm by Haddock, Mayer, and Sloanaker[4] in the initial application of NRL 50-ft antenna. They reported flux densities at 9.4 cm of Cassiopeia A, M 1, Cygnus A, the galactic center region, Centaurus A, M 87, and IC 443.[5] The flux densities together with those measured at longer wavelengths were found to be in rough accord with a λ^x law,[6] with the exception of a possible departure near 70 cm.[7] The spectra of the strongest of these sources were further extended to 3 cm by Haddock and McCullough in 1955.[8]

Subsequent studies of the strong discrete sources at 10 cm and 3 cm have been made by Razin and Pletchkov,[9] Kaydanovsky and Kardashev,[10] Jennison,[11] Broten and Medd,[12] and others.

The region in Sagittarius near the galactic center has recently been mapped by Drake[13] with a resolution of about 6′. Observing simultaneously at 68 cm, 21 cm, and 3.75 cm with the 85-ft antenna of the National Radio Astronomy Observatory, he has resolved four distinct centers of emission in the galactic plane arranged more or less symmetrically within 100 parsecs of the center. Professor Oort* has already commented on the correlation between this result and recent hydrogen line studies of the same region.

Ionized Hydrogen Regions.—The bright gaseous emission nebulae were identified as a new class of discrete radio source by Haddock, Mayer, and Sloanaker in 1954.[14] They reported detection at 9.4 cm of the Orion Nebula, as well as M 17, M 8, M 20, and an extended region of hydrogen emission nebulae near the galactic center. The cm wavelength emission intensity from these sources is found to be proportional to the intensity of their $H\alpha$ emission corrected for optical extinction, in accord with the interpretation of the radio emission as thermal radiation arising from free-free transitions in H II.[15] Further evidence for the thermal model is the radio spectrum of the Orion Nebula. The intensity is frequency independent at wavelengths shorter than about 20 cm and proportional to λ^{-2} at longer wavelengths at which the nebula is optically thick.

Thermal emission from planetary nebulae also appears to be a source of detectable radiation. In 1958 Drake and Ewen reported detection of 3.75 cm radiation associated with NGC 7293 and NGC 6853, the Helix and Dumbbell nebulae respectively.[16] Although this observation has not yet been corroborated at nearby wavelengths, it appears that cm wavelength observations will become a useful and independent tool in the study of the planetaries, for many of which the optical estimates of electron density are not of high precision.

Polarization of Discrete Sources.—Polarization of radio radiation from a discrete source was first detected by Mayer, McCullough, and Sloanaker at 3.15 cm.[17] They measured 7 per cent plane polarization for the Crab Nebula at a position angle

of 149° for the electric vector. A later measurement at 9.6 cm showed 3 per cent polarization at 142°.[18] The close agreement of these results with others at nearby wavelengths[19] and with the average optical position angle provides strong support for the synchrotron mechanism of emission from this source and indicates that Faraday rotation along the path between the Crab Nebula and the earth is small at cm wavelengths. Polarization of this source is not observed at longer wavelengths presumably because of the obscuring effect of enhanced Faraday rotation. Other strong sources studied at 10.2 cm show less than one per cent plane polarization.[19] Since Faraday rotation varies as λ^2, the cm region is uniquely suited for unambiguous polarization measurements of the discrete sources.

The Moon.—Measurements of continuum radiation at cm wavelengths from the moon and planets have yielded information on surface temperatures and on the characteristics of the surface material. The first such measurement was made by Dicke and Beringer[20] at 1.25 cm in 1945. They found the average disk temperature of the full moon to be 270°K. Piddington and Minnett[21] made an extensive series of observations at the same wavelength of the phase variation of the disk temperature. They reported the disk temperature of the new moon to be 145°K and the constant component of temperature to be 234°K. The latter is presumably the temperature of a well-insulated layer far below the surface. In contrast to infrared measurements in the 8–12 μ region, the microwave thermal radiation shows a phase lag of about 45° behind the lunar phase angle. The measurements were interpreted as indicating a dust layer of the order of a few mm in thickness and an effective depth of origin of the radiation of about 40 cm. Subsequent observations of thermal radiation from the moon as a function of lunar phase angle and during lunar eclipses have been made at 10 cm,[22] 3.2 cm,[23] and 8.6 mm.[24] Troitzky and Khaikin,[25] from an analysis of the 3.2 cm and optical data, calculate a constant component of radio temperature of 170°K and considerably less penetration than suggested in Ref. (21). The 8.6 mm measurements together with infrared data appear to suggest a dust layer one inch or more in thickness.[24] Additional and more precise observations seem necessary before a detailed and consistent model of the lunar surface is available. The radio measurements have recently been extended to 4.3 mm by Coates.[26] In this work scans of the lunar surface with 7′ resolution showed distinct features with various brightness characteristics.

The Planets.—Planetary radiation at radio wavelengths was detected in 1955 by Burke and Franklin,[26a] who identified Jupiter as the source of burstlike radiation in a narrow band around 15 m wavelength. Radiation resembling thermal emission was discovered by Mayer, McCullough, and Sloanaker[27, 28] in observations of Venus, Jupiter, and Mars at 3 cm wavelength. The results of these and subsequent measurements at cm wavelength are listed in Table 1. The temperature T is the apparent black body temperature of the disk. The notation $C + n$ refers to an observation made n days after inferior conjunction of Venus.

The cm wavelength observations of the planets have led to the discovery of at least two unexpected phenomena whose interpretation is still not clear.

(1) Venus has an apparent temperature of about 600°K, over twice the apparent infrared temperature of the region above the cloud surface and much higher than expected near the cloud surface on the basis of solar heating and a CO_2 atmosphere. The apparent temperature appears to be roughly wavelength independent, at least

TABLE 1

CENTIMETER AND DECIMETER WAVELENGTH OBSERVATIONS OF THE PLANETS

Planet	λ cm	T, °K	Comments	Ref.
Venus	3.15	620 ± 110	C − 48 to C − 30, 1956	(27)
	3.15	560 ± 73	C − 30 to C + 1, 1956	(27)
	3.4	518 ± 47	C + 15 to C + 36, 1958	(28a)
	3.37	575 ± 58	C + 80, 1958, (with maser)	(29)
	9.4	580 ± 160	C + 3 to C + 5, 1956	(27)
	0.86	410 ± 160	C + 1, 1958	(30)
Jupiter	3.15	145 ± 26	.....	(28)
	3.2 ± 0.2	177 ± 22	(With maser)	(45)
	3.75	210 ±	.....	(16)
	10.3	640 ± 85	.....	(31)
	21.0	2,500 ± 450	.....	(32)
	21.1	3,000 ± 1,300	Estimated from data in Ref. (34)	...
	21.4	3,500 ± 1,700	.....	(35)
	31	5,500 ± 1,500	.....	(33)
	68	70,000 ± 30,000	.....	(35)
Mars	3.15	218 ± 76	Opposition, 1956	(28)
	3.14	211 ± 28	Opposition, 1958, (with maser)	(45)
Saturn	3.75	...	Flux density 4×10^{-26} *w* m^{-2} $(cps)^{-1}$	(16)

between 0.86 and 10 cm, a behavior consistent with the interpretation that this is a real temperature, presumably that of a layer many kilometers below the cloud surface. The presence of such a high temperature would imply either a greenhouse mechanism of surprisingly high efficiency in the region of the cloud layer or the presence of an internal heat source.

(2) Jupiter is a source of high intensity radiation at centimeter and decimeter wavelengths. The radiation appears to be variable, with a suggestion in some observations of correlation with the rotation of the planet.[31] At least at decimeter wavelengths the radiation is probably not primarily of thermal or atmospheric origin. A tentative explanation proposed by Drake and Field[35] is that the nonthermal component is cyclotron or synchrotron radiation. The former envisages a belt of electrons surrounding the planet and trapped in magnetic fields between 150 and 1,000 oersteds. Much smaller magnetic fields can account for the observed radiation if the particles are relativistic. However, the required flux of relativistic particles seems higher than can be accounted for in cosmic rays and solar corpuscular emission.

Radar Astronomy.—Studies of radar return from the moon, first detected in 1946,[37] have yielded several different kinds of information. The measurement of the reflection coefficients as a function of wavelength gives information on the electrical characteristics of the lunar surface. From an analysis of the available data at wavelengths from 10 cm to 2.5 m, Senior and Siegel[38] have calculated the effective permittivity and conductivity of the surface to be 8.2×10^{-12} *farads/m* and 4.3×10^{-4} *mho/m* respectively. The details of echo fading of short pulses are capable, in principle, of providing information on the topography of the lunar surface. Echo fading at meter wavelengths, on the other hand, is in part due to the Faraday effect in the earth's ionosphere, and analysis of this effect has yielded a measure of the total electron content of the ionosphere.[39]

Precise range measurements at 10 cm wavelength using 2 μs pulses have recently led to a new estimate of the earth-moon distance with a precision of ±3 km.[40] In addition, the data provide indirect measurements of the mean equatorial radius of the earth and the mean horizontal parallax of the moon. The values ob-

tained show small but definite deviations from the accepted values as well as unexplained monthly and diurnal variations.

The only planet detected to date by radar is Venus. Its detection at 68-cm wavelength, reported by a group at Lincoln Laboratory,[41] required an 84-ft antenna, a receiver noise temperature of 170°K, and the use of automatic data processing equipment for statistical analysis of the result. Because of the marginal nature of the detection, little conclusive information could be obtained on the reflection characteristics of the planet or on the precise range.

NEW TECHNIQUES IN CENTIMETER WAVELENGTH RECEIVERS

Since future observations at cm wavelengths will probably rely heavily on recently-developed high sensitivity amplifiers as well as on antennas of increased area, it seems worthwhile to review briefly some of the new amplification techniques. The main part of this section will be devoted to a discussion of masers.

Two distinct approaches have been used to obtain greater sensitivity than is provided by the conventional superheterodyne receiver. The first consists of using a bandwidth much larger than the usual 5 or 10 mc. By an increase in bandwidth from 10 to 1,000 mc with the use of a traveling wave tube amplifier, Drake and Ewen[16] have obtained an order of magnitude decrease in output fluctuation level. With an integration time of 100 sec, they report an output noise level corresponding to 0.01°K in antenna temperature, even with a receiver noise temperature of about 4,000°K. The upper limit of receiver bandwith is determined by man-made interference and to a lesser extent by the desired spectral resolution.

The second approach is to decrease the receiver noise temperature by the addition of a low noise preamplifier such as a parametric device or a maser. With the use of a maser radiometer on the U. S. Naval Research Laboratory 50-ft antenna, Alsop, Giordmaine, Mayer, and Townes[42] have obtained a receiver noise temperature of 85°K, including the contributions of radiation from the ground and from the atmosphere. With an averaging time of 5 sec the output fluctuation level was 0.04°K. The maser preamplifier was developed at the Columbia Radiation Laboratory. Another solid-state maser amplifier with a system noise temperature of 170°K[41] was used in the radar detection of Venus and others have been or are being installed on antennas at Harvard College Observatory and the University of Michigan Observatory.

The NRL maser radiometer has already been described elsewhere in detail,[42] and the following is an outline of its characteristics. It consists of a superheterodyne receiver preceded by a three-level[43] ruby maser preamplifier, both mounted adjacent to the focus of the 50-ft antenna. The maser medium is a single crystal of ruby[44] maintained at liquid helium temperature in a multiply-resonant microwave cavity, in a magnetic field of about 3,500 oersteds. Under these conditions four energy levels separated by about 1 cm^{-1} are accessible to the Cr^{+3} paramagnetic impurity atoms. The equilibrium population distribution of the levels is disturbed by continuous saturation of the first (ground) level and the third level by power at 1.3 cm wavelength. In this situation the population of the third level can be made higher than that of the second, and amplification by stimulated emission is possible at the difference frequency, in this case, 3 cm wavelength. The microwave cavity is of such dimension as to be resonant at both the "pump-

ing" frequency and the amplification frequency when completely filled with ruby. The product of (voltage gain) × (bandwidth) is about 50 mc in this device. This quantity remains constant as the gain of the amplifier is changed by varying the strength of the coupling between the input waveguide and the cavity.

The inherent noise in such a radiometer is quite low. Almost all of the 85°K noise arises from thermal radiation from ancillary components and from the ground. The contribution to the noise from spontaneous emission, which determines the ultimate sensitivity of an ideal maser amplifier, is only about 3°K.

Liquid helium is necessary in this and other masers suitable for radio astronomy at present, in order to provide a large population difference for the amplifying transition and consequently a useful gain-bandwidth product. A ruby maser has been operated at dry ice temperature, but only at great expense of gain and bandwidth.[46] In principle, however, liquid helium temperature is not essential to attain low noise temperature, and in future we may expect masers for radio astronomy that can be used with more convenient coolants.

The signal from the feed horn at the antenna focus is introduced to the reflection-type maser cavity through a ferrite circulator, which also transmits the amplified signal to the superheterodyne receiver and isolates the maser cavity from noise originating in the mixer crystals. The maser input is switched at 30 cps between the feed horn and a "reference" horn of broad beamwidth pointing at the sky. An adjustable attenuator in the reference branch allows the two signals to be balanced to within a few tenths of a degree, so that the receiver acts as a null system. With the antenna stationary, the rms gain variation is of the order of 0.01 db per minute.

The addition of the maser preamplifier provided an improvement in sensitivity of about 12 compared with the superheterodyne receiver alone, accepting both image bands. The noise temperature of 85°K can probably be reduced an order of magnitude by improvements in component and maser design and by precautions to reduce antenna spillover. A 5.4 cm traveling wave maser with horn antenna has been operated by de Grasse, Hogg, Ohm, and Scovil[44a] with a total noise temperature of 18°K including contributions from spillover. With more efficient design antenna temperature sensitivities of the order of 10^{-3}°K should soon be feasible at cm wavelengths. The upper limit of useful receiver sensitivity imposed by receiver gain fluctuations, fluctuations in atmospheric absorption and background radiation, and source confusion in the case of all but the largest antennas probably occurs close to this sensitivity.

OBSERVATIONS USING THE MASER

The following is a summary of some of the results obtained by Alsop, Giordmaine, Mayer, and Townes with the use of the maser radiometer on the NRL 50-ft antenna.[45, 47]

Venus.—The increased sensitivity of the maser permitted a measurement of the apparent temperature of Venus 80 days after the conjunction of 1958 (Table 1). At that time the antenna temperature due to Venus was 0.45°K. In the 80 days following conjunction the fraction of the visible disk illuminated by the sun increases from less than 1 per cent to about 55 per cent. During this period there appears to be no conclusive change in the apparent disk temperature. There is, however, a suggestion of a slightly lower night temperature after conjunction than

before. Such an effect, if corroborated, would be consistent with retrograde rotation of Venus. Retrograde rotation has also been suggested, although inconclusively, by spectroscopic observations. The approximate equality of the day and night temperatures, as measured by both infrared and microwave techniques, indicates that the rotation period is appreciably different from the period of revolution, and in principle can be used to set a lower limit to the rotation period.

Jupiter.—Observations of Jupiter were made on 18 days in the period April, 1958 to February, 1959 in the wavelength range 3.0 to 3.4 cm. From a total of 153 flux measurements the average apparent temperature of the visible disk was found to be 177 ± 22°K. The observations are summarized in Figure 1. Each point represents an average of 9 measurements of peak antenna temperature on a particular day. The relative mean error is the average statistical error of each point. The absolute mean error includes possible systematic errors common to all the points, for example the uncertainty in antenna calibration. In this work individual drift curves, representing a peak antenna temperature of about 0.4°K, were analyzed with a template and the peak temperatures combined to form the daily averages.

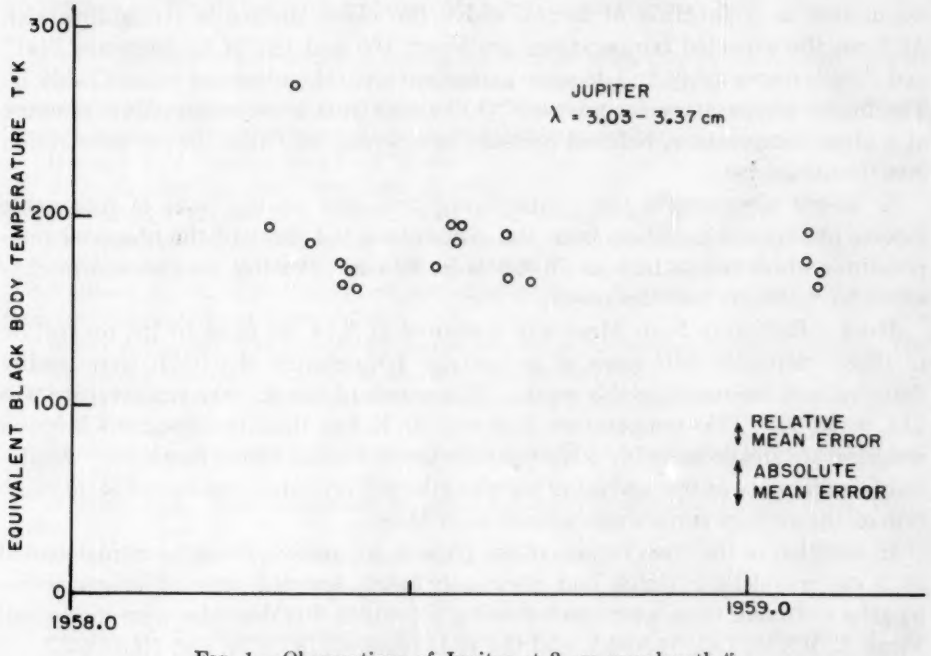

FIG. 1.—Observations of Jupiter at 3 cm wavelength.[45]

Between 3.2 and 3.4 cm wavelength there is a decrease in apparent temperature with increasing frequency of 12 ± 11°K/kmc. The average temperature during this period appears to be significantly higher than the average temperature measured in 1957 (Table 1). The beginning of our observation period, April 16, 1958, immediately preceded an outbreak of activity in the south equatorial belt, between April 18 and April 26.[48] The anomalously high temperature recorded April 30 to May 1, about 270°K, refers to the hemisphere of Jupiter containing the head

of the outburst. One can conclude that there are detectable fluctuations in the 3 cm radiation temperature of the planet, with the suggestion that changes in the apparent temperature may be correlated with changes in the appearance of the planet.

No correlation was observed between the apparent temperature and the rotation of the planet nor between apparent temperature and solar activity as measured by the 10 cm solar flux intensity. There was no detectable linear polarization.

The magnitude of the 3 cm radiation can be interpreted[36, 45] entirely in terms of thermal radiation from NH_3 known from spectroscopic measurements to be present in the atmosphere. NH_3 radiates in the cm region through the pressure-broadened inversion line at 1.28 cm wavelength. Let us assume that H_2, He, and CH_4 are present in the atmosphere above the cloud surface in relative abundances corresponding to the mixtures "(a)" and "(b)" proposed by Kuiper.[49] We assume further that the region above the cloud surface has an adiabatic temperature gradient and is saturated with NH_3. These assumptions, together with the measured NH_3 and CH_4 abundances, fix the temperature, pressure, and composition distribution above the cloud surface. The apparent temperature as a function of wavelength can then be calculated from the NH_3 absorption coefficient, whose calculation as a function of height above the cloud surface is straightforward. At 3 cm the expected temperatures are about 160 and 183°K for mixtures "(a)" and "(b)", respectively, in adequate agreement with the observed value (Table 1). The higher temperature for mixture "(b)" arises from lower atmospheric pressure at a given temperature, reduced pressure broadening, and thus deeper penetration into the atmosphere.

At longer wavelengths the temperatures predicted on the basis of reasonable models of thermal radiation from the atmosphere fall short of the observed temperatures which rise as high as 70,000°K at 68 cm. Possible nonthermal mechanisms have already been discussed.

Mars.—Radiation from Mars was measured at 3.14 cm close to the opposition of 1958. Separate drift scans at an antenna temperature of 0.08°K were readily detected and averaged in this work. The apparent black body temperature was 211 ± 28°K. This temperature is about 50°K less than the apparent infrared temperature and presumably is the temperature of a cooler subsurface layer. Analysis of similar data at this and other wavelengths will provide a new source of information on the average surface characteristics of Mars.

In addition to the observations of the planets, a number of sources were detected at 3 cm wavelength which had previously been detected only at longer wavelengths. Among these were the following, for which flux densities were measured: Virgo A, IC443, IAU 16N04A, and the H II regions M8, M20, and NGC 6357.

A thorough search was made for several planetary nebulae, including NGC 6853, NGC 7009, and NGC 7293. Preliminary analysis of the data indicates that an upper limit of about 0.1°K can be set for the antenna temperatures associated with these objects.

DIRECTIONS OF FUTURE CENTIMETER WAVELENGTH RESEARCH

In conclusion, I would like to list briefly some of the predictable areas in which cm wavelength research is likely to proceed in the next few years. Much of this

work will require the combination of low noise amplifiers and the largest antennas.

(1) Extension of the spectra of the brighter sources to the mm region, and the spectra of many of the weaker sources to 10 and 3 cm; systematic surveys at 10 and 3 cm.

(2) Precise position measurements, facilitating efforts toward optical identification.

(3) Search for spectral lines, including the hyperfine transition in He^3 II at 3.46 cm and the fine structure transition in metastable H I at 3.03 cm.[50]

(4) High resolution studies of size and brightness distribution of extended sources.

(5) Sensitive polarization measurements on the discrete sources, including the planets.

(6) Study of ionized hydrogen regions including the planetary nebulae.

(7) Study of problems connected with the planets: for example, the nature of the cloud layer and surface of Venus, the rotation period of Venus, the radiation mechanism on Jupiter, and the structure of planetary atmospheres as accessible for example through the radiation profile of the Jovian NH_3 inversion line and the absorption profile of the terrestrial lines of O_2 in the mm region.

* One of four papers presented in a symposium on radio astronomy at the Autumn Meeting of the National Academy of Sciences at Indiana University, November 17, 1959. Other papers from the symposium, by J. H. Oort, and R. Minkowski, appear on pages 1–19 (vol. 46). The remaining paper, by Alan Maxwell, will be published upon receipt.

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THE CONTROL OF THE FORMATION OF SPECIFIC PROTEINS IN BACTERIA AND IN ANIMAL CELLS

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In 1953, Monod and Cohen-Bazire as well as Cohn, Cohen, and Monod showed that an amino acid may repress the *last* enzyme in the biochemical pathway which leads to the formation of that amino acid.

Four years later, H. J. Vogel found that arginine, when added to a growing bacterial culture, represses the formation of acetyl ornithinase (one of the *early* enzymes along the biochemical pathway leading to arginine), which converts acetyl-ornithine into ornithine. This observation provided rather strong circumstantial evidence in favor of the view that enzyme repression may be part of the normal regulatory mechanism of enzyme formation in bacteria.

Subsequently, it occurred to Werner Maas that inducers which enhance the formation of an enzyme when added to a growing bacterial culture may perhaps be capable of doing so *only* because there is a repressor present in the cell, and that the inducer might perhaps do no more than inhibit some enzymes which are involved in the formation of the repressor. Thus the inducer would enhance the *formation* of an enzyme only because it reduces the concentration of the repressor in the cell. (Oral communication, April, 1957.)

At that time, the best investigated case of enzyme induction was the induction of the enzyme β-galactosidase. Milton Weiner helped my understanding of the induction of this enzyme greatly by pointing out that its induction must be considered in conjunction with the biochemical pathway leading from galactose to glucose-1-P. (Oral communication, January, 1957.)

The induction of β-galactosidase in a culture of bacteria growing on succinate or lactate, as the carbon source, is inhibited by adding glucose to the growing culture. From this I was led to infer that some metabolite, intermediate in the sequence of metabolites lying between galactose and glucose-1-P, is the precursor of the repressor of β-galactosidase. Further, in line with the above quoted suggestion of Werner Maas, I was led to surmise that certain galactosides may induce β-galactosidase, by inhibiting enzymes which lie on the biochemical pathway between glucose 1-P and the intermediate metabolite that may be the precursor of the repressor of this enzyme.

I believe that some such galactosides may in fact inhibit one of these enzymes and this may explain, in part, why the rate of formation of β-galactosidase rises faster than linearly with the intracellular concentration of the inducing galactoside. However, since I was not able to explain on any similar basis the induction of the degradative enzymes by their substrate, I was led to assume that the inducer must be able to exert an effect which goes beyond inhibiting the formation of a repressor. In particular, I was led to believe that a repressor may reduce the rate of formation of the enzyme by combining with an enzyme molecule which is still attached to its enzyme-forming site, and that it may thereby somehow prevent the attached enzyme molecule from leaving its enzyme-forming site.† According to this view the

repressor combines with a specific site, the controlling site, of the "attached" enzyme molecule, and an inducer may then enhance the formation of the enzyme by competing with the repressor molecule for this site.

I was further led to believe that the repressor of the enzyme β-galactosidase might be a molecule composed of two moieties. One of these (which we may call the metabolite moiety) might be a galactoside and the other (which we may call the *R* moiety) might perhaps be a polynucleotide. Certain galactosides would then enhance the formation of the enzyme β-galactosidase by competing for the controlling site of the attached enzyme molecule with the metabolite moiety of the repressor.

A model which was based on this mechanism of induction and repression of enzyme formation in bacteria was presented by me in a paper given at the annual meeting of the German Chemical Society in Berlin (Oct. 7, 1957) and in lectures, given in the subsequent six months, at various institutions actively interested in the problem of enzyme induction.

The model appeared to be capable of accounting not only for enzyme repression but also for some aspects of antibody formation in mammals. It was not then clear, however, whether it might provide a convincing explanation for the phenomenon of lasting immunity. Such lasting immunity manifests itself in the secondary antibody response which may be elicited in mammals such as the rabbit when it is given an injection of an antigen by which it had been immunized earlier. This secondary response can be elicited even if the second injection of the antigen follows the first injection of the antigen after a very long time interval and thus it involves a "memory" which fades away only very slowly.

In the postwar years the study of inducible enzymes received its impetus chiefly from Jacques Monod's studies of the induction of the enzyme β-galactosidase, at the Institut Pasteur in Paris. The induction of this enzyme turned out to be a very complex phenomenon, however, and until recently it could only be inferred that it involved enzyme repression.

Two years ago, when the model here discussed was first presented, it was supported only by scattered experimental facts. In particular the tenet that the *R* moiety might be a polynucleotide was based on rather tenuous circumstantial evidence.

In the last two years, however, very considerable progress has been made in the study of enzyme induction and enzyme repression. Thus an experiment by Arthur Pardee, Francois Jacob, and Jacques Monod has provided evidence in favor of the view that the phenomenon of enzyme repression may play a major role in the induction of β-galactosidase. Recent experiments performed by Luigi Gorini, at the Medical School of Harvard University, and by George Cohen, Francois Gros, Francois Jacob, Werner Maas, Jacques Monod, and Arthur Pardee, at the Institut Pasteur, support the view that enzyme repression may be the key to the understanding of the phenomenon of enzyme induction in general.

One of these recent experiments shows that a bacterial gene which is responsible for the repression of the enzyme β-galactosidase may exert its effect not by causing the synthesis of an enzyme, but rather by causing the synthesis of a molecule which is not a protein. This remarkable finding is consistent with our notion that this

gene might cause the formation of the R moiety of the repressor of β-galactosidase and that this R moiety might be a polynucleotide.

Apparently, the results of all these newer experiments are, so far, still consistent with the model formulated two years ago, and, in this limited sense, they lend support to the model.

REPRESSION AND INDUCTION OF THE FORMATION OF ENZYMES IN BACTERIA

1. *Enzymes Leading to Glucose-1-P.*—There are numerous repressible enzymes involved in the biochemical pathways which lead from a carbon source to glucose-1-P. In general, the formation of such enzymes is inhibited by glucose. This is quite consistent with the views on enzyme repression here adopted and may be readily understood on the basis of the considerations presented by Neidhardt and Magasanik (1956).

I propose to skip the discussion of this large class of enzymes in favor of discussing two other large classes of enzymes which we may designate as "special anabolic enzymes" and "degradative enzymes."

2. *Special Anabolic Enzymes.*—The enzymes which lie along the biochemical pathways that lead to the formation of an amino acid, a purine, or a pyrimidine, we shall designate as "special anabolic enzymes." We shall single out the enzymes, E_i, involved in the biosynthesis of the amino-acid arginine as being representative for the enzymes of this class.

One of these enzymes, ornithine transcarbamylase, which converts ornithine into citrulline, has been studied by Luigi Gorini and Werner Maas. In a certain strain of coli, the formation of this enzyme may be repressed by adding arginine to a growing bacterial culture. If the intracellular concentration of arginine is lowered in a mutant which cannot convert citrulline into arginine by growing it at a slow rate in a chemostat, with arginine as a controlling growth factor, then the rate of production of the enzyme is raised by a factor of about 25.

We shall refer to enzymes whose rate of production can be thus raised as "boostable" enzymes.

The high rate of enzyme production which may be obtained in the case of such a "boostable" enzyme by lowering the intracellular concentration of a controlling metabolite may represent the "full rate" at which an enzyme-forming site is capable of synthesizing the enzyme.

There are other strains of coli in which the rate of formation of ornithine transcarbamylase cannot be boosted by lowering the intracellular concentration of arginine or any other *known* metabolite. We shall not discuss these "unboostable" strains in the present paper.

We do not assume that the molecule responsible for the repression of ornithine transcarbamylase in the "boostable" strains is arginine itself. We rather assume that the repressor REP_i of the enzymes E_i is a composite molecule which consists of two moieties. One of these, the "metabolite" moiety, is arginine; the other is a moiety which we shall designate by R_i and to which we shall refer as the R-moiety. Thus we may write for the chemical formula on the repressor REP_i

$$REP_i = (\text{arginine} - R_i)$$

As stated above, the R moiety of such a repressor molecule might possibly be a polynucleotide.

Our model for the repression of the enzymes E_i, involved in the biosynthesis of arginine, is as follows:

We assume that the polypeptide chain of the enzyme molecule is synthesized along a specific enzyme-forming site, which determines the amino-acid sequence of the polypeptide. This polypeptide folds up to form the enzyme molecule, but this molecule remains attached, perhaps through a covalent bond, to the enzyme-forming site. If there are repressor molecules present in the cell which are specific for this enzyme, then the metabolite moiety of a repressor molecule may reversibly combine with the controlling site of the attached enzyme molecule (and the R-moiety might perhaps reversibly combine with the purine and pyrimidine base of the enzyme-forming site itself). We postulate that an "attached" enzyme molecule, which is so combined with a repressor molecule, cannot leave its enzyme-forming site and accordingly the formation of the enzyme may thus be repressed.

Concerning the mechanism of the action of the repressor we may assume—*pour fixer les idées*—that there is some universal enzyme U present in the cell which can split the covalent bond that ties newly-formed enzyme molecules to their enzyme-forming site. The repressor molecule, when it is combined with an attached enzyme molecule, sets up a "steric hindrance" and may thus prevent the universal enzyme U from splitting the covalent bond.

We postulate that the enzymes E_i, lying along a biochemical pathway that leads to an amino acid, have two specific combining sites, the "catalytic" site and the "controlling" site.

In the case of an enzyme which catalyzes an early step in the biosynthetic pathway, leading to an amino acid, a purine or a pyrimidine, there need be little chemical resemblance between the substrate of the enzyme and the amino acid, the purine, or the pyrimidine, which lies at the end of the biosynthetic pathway and which may be capable of repressing the formation of the enzyme. The substrate has a specific chemical affinity to the catalytic site of the enzyme, and the end product of the biosynthetic chain, the amino acid, the purine, or the pyrimidine, has a specific affinity to the controlling site. Therefore in the case of these "early" enzymes, the combining specificity of the catalytic site and of the controlling site might be quite different.

In the case of the enzymes which lie towards the end of the biochemical pathway, however, the substrate of the enzyme is likely to be a chemical analogue of the "end product" and we may expect it therefore to have specific chemical affinity, not only for the catalytic site but also for the controlling site of the enzyme.

3. *Degradative Enzymes E_j.*—The enzymes involved in the degradation of tryptophane to β-keto-adipic acid by *Pseudomonas fluorescens* may be taken to be representative for another large class of enzymes, the "degradative enzymes." There are seven enzymes involved in the degradation of tryptophane, and the formation of each of them can be enhanced by adding the substrate of the enzyme to the growing bacterial culture.

There is a vast array of compounds which can be oxidized by bacteria such as *P. fluorescens*. R. Y. Stanier estimated their number at more than 50 and estimated that at least 200 enzymes participate in their degradation. The formation of each of these enzymes may be expected to be enhanced by adding its substrate

to the growing bacterial culture, and we may therefore regard these "degradative enzymes" as "inducible" enzymes.

We postulate that the degradative enzymes E_j also have two specific combining sites, a catalytic site and a controlling site. Since in the case of the degradative enzymes the substrate of the enzyme is an inducer of the enzyme, we assume that the substrate of such a degradative enzyme has a substantial chemical affinity for the controlling site as well as the catalytic site. In the case of the degradative enzymes, E_j, we may therefore expect the two combining sites to be similar in their combining specificity for any given metabolite M.

The metabolite moiety of the repressor of a degradative enzyme might be either the substrate itself or else a metabolite further down the degradative pathway which is still a chemical analogue of the substrate.

If the metabolite moiety of the repressor is the substrate itself, and if the concentration of the repressor rises more slowly with increasing intracellular concentration of the substrate than the concentration of the substrate, then the substrate must be an inducer of the enzyme because it competes with the repressor for the attached enzyme molecule.

The concentration of the repressor would rise more slowly than the concentration of the substrate, for instance, if the limiting factor for the rate of production of the repressor were the rate of production of the R moiety of the repressor.

4. *The Coupling Enzymes C_i or C_j.*—We postulate that there may be present in the bacterial cells a class of enzymes to which we may refer as coupling enzymes, C_i and C_j, which couple a specific R moiety, R_i and R_j, to the metabolite M_i and M_j, and thus form the repressor REP_i and REP_j respectively.

Within the class of the degradative enzymes E^j, the corresponding coupling enzymes C_j might be close to being saturated with respect to the metabolite moiety of the repressor. With increasing intracellular concentration of the substrate, the concentration of the repressor would then rise more slowly than the concentration of the substrate and on this basis one may then expect the substrate to be an inducer of the enzyme.

If a compound $\bar{M}$ is a chemical analogue of the metabolite moiety M of the repressor REP, and if the cell cannot transform it into the metabolite moiety M, then such a compound may reduce the concentration of the repressor by inhibiting the coupling enzyme C and thereby enhance the formation of the enzyme E.

Equations Describing Our Model for Enzyme Repression and Induction.‡—In a bacterial culture, growing at a fixed rate, the total repressor concentration ρ_0, that establishes itself in the stationary state, is proportional to the rate at which the repressor molecules are formed.

We may compute ρ, the concentration of the free repressor molecules, from ρ_0, the total concentration of the repressor molecule J by writing

$$\rho = \rho_0 - z\frac{\rho/K}{1 + \rho/K} \tag{1A}$$

where K designates the equilibrium constant for the dissociation of the repressor molecule from the controlling site of the "unattached" enzyme molecules and z the concentration of the enzyme E in the cell.

In (1A) the first term represents the total concentration of the repressor in the

cell and the second term represents the concentration of those repressor molecules which are combined with the controlling site of an "unattached" enzyme molecule. For the sake of keeping our formulae simple we are disregarding here the fact that the repressor may also combine with the catalytic site of the "unattached" enzyme molecules. Accordingly (1A) must be amplified when this becomes relevant to the issue considered.

We may write (1A) also in the form of (1B)

$$z = \rho_0 \left(1 - \frac{\rho}{\rho_0}\right) + K \left(\frac{\rho_0}{\rho} - 1\right) \tag{1B}$$

We shall designate by τ the average time for which a newly-formed enzyme molecule remains tied to its enzyme-forming site, when it is *not* combined with a repressor molecule at its controlling site. We shall assume that τ is large compared to the time that it takes for the polypeptide to be formed and to fold up to form the attached enzyme molecule. In these circumstances we may say that there is practically always an enzyme molecule attached to the enzyme-forming site, and this enzyme molecule is either combined with a repressor molecule or it is not.

On the basis of our model we may then say that the rate of formation of an enzyme, when it is limited by the presence of a repressor molecule in the cell, is given by

$$\text{rate} = q/\tau \text{ per enzyme-forming site, per unit time} \tag{2}$$

where q is the probability that the attached enzyme molecule is *not* combined at its controlling site with a repressor molecule.

We may write for this probability q, in the presence of a repressor *REP* and an inducer $\bar{M}$

$$q = \frac{1}{1 + \mu/K_{\bar{M}}^* + \rho/K^*} + \frac{\mu/K_{\bar{M}}^*}{1 + \mu/K_{\bar{M}}^* + \rho/K^*} \tag{3}$$

μ and ρ are the intracellular concentrations of the inducer $\bar{M}$ and the free repressor *REP*, respectively; $K_{\bar{M}}^*$ and K^* are the equilibrium constants, for the reversible dissociation of the inducer molecule $\bar{M}$ and the repressor molecule *REP*, respectively, from the controlling site of the attached enzyme molecule.

In (3) the first term gives the fraction of the attached enzyme molecules which are not combined at their controlling site with either a repressor molecule *REP* or an inducer molecule $\bar{M}$. The second term represents the fraction of the attached enzyme molecules which are combined at the controlling site with an inducer molecule $\bar{M}$.

Formula (3) holds true if the average time that it takes an inducer molecule to dissociate from the controlling site of the attached enzyme molecule is short in comparison with τ.

We may write from (2) and (3) for the rate of enzyme formation per enzyme-forming site per unit time

$$\text{rate} = \frac{1}{\tau} \frac{1 + \mu/K_{\bar{M}}^*}{1 + \mu/K_{\bar{M}}^* + \rho/K^*} \tag{3A}$$

The concentration of an enzyme in a bacterium that grows at a fixed rate is pro-

portional to the rate at which the enzyme is formed. Accordingly, we may write for z, the intracellular concentration of the enzyme

$$z = z^* \frac{1 + \mu/K_{\bar{M}}^*}{1 + \mu/K_{\bar{M}}^* + \rho/K^*} \tag{4}$$

where z^* is the concentration which the enzyme might attain in the cell, in the absence of any repressor.

In the absence of the inducer $\bar{M}$ we may write

$$z = z^* \frac{1}{1 + \rho/K^*}. \tag{5}$$

Equations (4) and (5) give the concentration of the enzyme in the cell independent of how many enzyme-forming sites are present in the cell which synthesize the same enzyme. In (4) and (5), z^* represents the enzyme concentration which is obtained from these formulae when ρ, the repressor concentration, becomes zero.

It should be noted, however, that when the repressor concentration becomes very small and the concentration of the enzyme becomes correspondingly large, the repressor concentration may cease to be the limiting factor for the rate of enzyme production and something else may become rate-limiting. For this reason the enzyme concentration in the cell might not actually reach the value z^*, if the repressor concentration goes to zero.

We may now introduce into our formulae the repression factor λ which is defined by

$$\lambda = \rho_0/K^*.$$

We may then write (5) in the form

$$z = z^* \frac{1}{1 + \lambda\rho/\rho_0} \tag{5A}$$

and this we may also write in the form

$$\rho = \frac{\rho_0}{\lambda} \frac{1 - z/z^*}{z/z^*} \tag{5B}$$

We shall throughout the rest of our discussion invariably assume that we have

$$z^* \gg \rho_0. \tag{6}$$

The Simplified Equations.—For the enzymes E for which we may write

$$K \gg z^*$$

the second term in (1A) can be neglected and we may then write

$$\rho = \rho_0.$$

In this case (4) and (5) may be written in the form of

$$z = z^* \frac{1 + \mu/K_{\bar{M}}^*}{1 + \mu/K_{\bar{M}}^* + \rho_0/K^*} \tag{7}$$

and

$$z = z^* \frac{1}{1 + \rho_0/K^*} = \frac{z^*}{\lambda + 1} \tag{8}$$

When these simplified equations hold, then the rate of enzyme formation is independent of the concentration of the enzyme in the bacterium. Accordingly, if an inducer is added to a growing bacterial culture at a given point in time, the rate of enzyme formation will rise to a new value at the time when the inducer is added and from then on it will remain constant. This could be verified by studying the kinetics of the induction of the enzyme.

It may be seen from (7) that if the presence of the inducer $\bar{M}$ does not affect ρ_0, the concentration of the repressor molecules in the cell, then the rate of formation of the enzyme cannot rise any faster than linearly with the intracellular concentration of the inducer.

If it is found that the enzyme concentration rises faster than linearly with the intracellular concentration of the inducer, we may then expect either that the inducer inhibits an enzyme involved in the formation of the metabolite moiety of the repressor or that it inhibits the enzyme C which couples the metabolite moiety of the repressor to the R moiety of the repressor, or that it does both.

Predictions Based on the "Simplified" Equations.—On the basis of the model as described by the above given simplified equations we may expect the following:

(a) If the cells of a bacterial strain are incapable of converting $\bar{M}$ (a chemical analogue of the repressor's metabolite moiety) into M (the repressor's metabolite moiety itself), and if the concentration of M is fixed, then the chemical analogue $\bar{M}$ may enhance the formation of the enzyme, provided that it can get into the bacterial cell.

The chemical analogue $\bar{M}$ may be an inducer of the enzyme in such a bacterial strain, either because it competes with the metabolite moiety of the repressor for the controlling site of the enzyme molecule which is attached to its enzyme-forming site, or because it competes with the metabolite M for the coupling enzyme C which joins the metabolite moiety to the R moiety of the repressor, or for both of these reasons.

In the case of the bio-synthetic pathway leading to arginine the substrate of a *late* enzyme is a chemical analogue of arginine. Accordingly, we may expect such a substrate to induce the enzyme in mutant bacterial strains which cannot convert the substrate into arginine. Thus we may expect the enzyme ornithine transcarbamylase, which converts ornithine into citrulline, to be inducible by citrulline, in a mutant strain which cannot convert citrulline into arginine.

Luigi Gorini has observed that ornithine induces the enzyme ornithine transcarbamylase in such a mutant, if the intracellular concentration of arginine is kept *moderate* by growing the bacterium *at a fast rate* in a chemostat with arginine as a controlling growth factor. (Oral communications, 1959.) (If the intracellular concentration of arginine were kept low by growing the bacterium at a slow rate in the chemostat, then the enzyme level would be boosted to a high value and the inducing effect of ornithine would not be observable.) Since this mutant converts ornithine into citrulline, Gorini's observation is consistent with the views here presented.

(b) There might exist mutants in which the R moiety of the repressor is produced, but it is produced at such a low rate that its production may be the limiting factor for the production of the repressor when the intracellular concentration of the metabolite moiety of the repressor is not too low. On the basis of (7), we may then say that in such a mutant the formation of the enzyme should be enhanced by adding the repressor's metabolite moiety to the growing bacterial culture. Accordingly, for such a mutant the metabolite moiety of the repressor may be an inducer of the enzyme.

Thus, in such a mutant, arginine (for instance) should be an inducer of the enzyme ornithine transcarbamylase and it should be an inducer of this enzyme for one reason only, i.e. because it may compete with the repressor for the controlling site of the attached enzyme molecule.

(c) There may exist mutants which are not capable of producing the R moiety of the repressor. In such a mutant the enzyme may be produced at the full rate and accordingly the enzyme level would be very high. In such a mutant it should not be possible to repress the enzyme by adding the metabolite moiety of the repressor to the growing bacterial culture. Mutants of this type may be designated as "absolute constitutive strains."

Both Luigi Gorini and Werner Maas have obtained from a strain of coli, in which the enzyme ornithine transcarbamylase is repressible by arginine, mutants in which the enzyme is always maintained at a high level and is not repressible by arginine. (Oral communications, 1959.) Conceivably these might be mutants in which the R moiety of the repressor is not formed, i.e., they might be absolute constitutive strains.

The Case of $z^* \gg K$. — We may postulate here, for the sake of argument, a set of constants for "wild type" bacteria which might be as follows:

$$z^* = 10^{-4} \text{ mol/l}, \qquad \rho_0 = 10^{-6} \text{ mol/l};$$
$$K = 10^{-5} \text{ mol/l}; \qquad K^* = 10^{-10} \text{ mol/l}$$

The postulated value of $z^* = 10^{-4}$ mol/l would mean that a fully boosted enzyme amounts to about 10 per cent of the cell proteins.

The value of $\rho_0 = 10^{-6}$ mol/l postulated would be consistent with the assumption that there may be one thousand different repressors present in a bacterium and the assumption that the R moiety of these repressors is a polyribonucleotide of a molecular weight of about 2,000, without having to assume a larger amount of soluble *RNA* in the bacterium than is usually found in bacteria.

The postulated value of $K = 10^{-5}$ mol/l means that the free energy change for the combination of the repressor with the controlling site of an unattached enzyme molecule is about $\Delta F = 7{,}000$ cal/mol. This appears to be a reasonable value if we assume that only the M moiety of the repressor has a specific chemical affinity to the controlling site of the unattached enzyme molecule.

The postulated value of $K^* = 10^{-10}$ mol/l corresponds to a free energy change of $\Delta F = 14{,}000$ cal/mol for the combination of the repressor with the enzyme molecule that is attached to its enzyme-forming site. This appears to be a reasonable value if we assume that the M moiety of the repressor has a specific chemical affinity to the controlling site of the enzyme molecule and that the R moiety of the repressor

has a specific chemical affinity to the purine and pyrimidine bases of the enzyme-forming site itself.

For the above postulated set of constants we have

$$z^* \gg K$$

and accordingly the simplified equations do not strictly speaking hold.

Nevertheless, for this set of constants, ρ, the free repressor concentration, would be very close to ρ_0, because the enzyme is strongly repressed. We may write for the repression factor λ, for our set of constants,

$$\lambda = \rho_0/K^* = 10^4$$

and we have accordingly for the above postulated set of values

$$\frac{z^*}{K(\lambda + 1)} = 10^{-3} \ll 1.$$

In these circumstances ρ may be close to ρ_0.

Limiting ourselves, for the moment, to a set of constants where we have

$$K \gg \rho_0$$

we may now say the following:

If we have to deal with a *mutant* in which the repressor is produced at a very low rate, so that the repression factor λ is small, and if we have

$$\frac{z^*}{K\ (\lambda + 1)} > 1,$$

then ρ, the free repressor concentration, may be much smaller than ρ_0 and, accordingly, the enzyme level in the cell may then be rather high. This may be seen from (1A) as follows:

We may write for the second term in (1A)

$$z\,\frac{\rho/K}{1 + \rho/K} > \frac{z^*}{\lambda + 1}\,\frac{\rho/K}{1 + \rho/K} \approx \frac{z^*}{\lambda + 1}\,\frac{\rho}{K}$$

and thus we obtain from (1A)

$$\frac{\rho_0}{\rho} > 1 + \frac{z^*}{K(\lambda + 1)}$$

and from (5A) we obtain

$$z > \frac{1}{1 + \lambda/\{1 + z^*/[K(\lambda + 1)]\}}\,z^*.$$

Accordingly, strains of this type may maintain an enzyme level which may be high and which might come close to the fully boosted enzyme level z^* of an "absolute constitutive mutant."

Paraconstitutive Enzymes.—If for an enzyme we have

$$\rho_0 \gg K$$

and if the other constants fall within certain ranges, then the concentration of such

an enzyme may be maintained in the cell either at a low stable value or at a high stable value—in the absence of any inducer.

An enzyme for which this holds we shall designate as "paraconstitutive."

The concentration of such a paraconstitutive enzyme may be maintained indefinitely at a low level in a proliferating cell culture. But, if once the enzyme concentration is raised to a sufficiently high level and maintained there long enough to establish a stationary state, from there on a high rate of production of the enzyme may be permanently sustained—even in the absence of an external, or internal, inducer. Further, when such a cell, which sustains a high enzyme level, divides, then the daughter cells which it generates will also sustain a high enzyme level. Thus the property of sustaining a high enzyme level is hereditary even though the inheritance is not genic.

It is not possible to say whether paraconstitutive enzymes exist in bacteria. If they did exist, it would be somewhat difficult to recognize them, because if they have arisen in the normal course of evolution then it is likely that by now the enzyme would be sustained at a high level, and the paraconstitutive mutant would thus give the appearance of a constitutive mutant.

It might well be, however, that the condition $\rho_0 \gg K$ is not fulfilled for any of the bacterial enzymes. Perhaps, in order to have this inequality hold for an enzyme, it is not sufficient for the controlling site of the enzyme to have a specific combining affinity for the metabolite moiety of the repressor, but it is also necessary for it to have a specific combining affinity for one or more of the units which constitute the R moiety of the repressor.

Paraconstitutive Enzymes and Cellular Differentiation.—Even though the inherent instability of the enzyme-forming system which is described by our equations might play no role in bacteria, it may still be of great interest. It is conceivable that it might play a major role in certain types of cellular differentiation, in higher organisms such as mammals.

There is no need to assume (and in the case of amphibia there may indeed be reason to doubt) that the early cellular differentiation involved in the formation of ectoderm, mesoderm, and endoderm, is of this type. Still, cellular differentiation in the later phases of embryonal development, where an organizer is involved, might conceivably be based on the inherent instability of the enzyme-forming system here discussed. It is not possible, however, to substantiate this at present on the basis of available experimental evidence, and it may be very difficult to substantiate it even through experiments devised for the purpose.

Experimental confirmation of the view that the model here discussed may provide the molecular basis of a certain type of cellular differentiation in mammals might, however, be obtained in the case of antibody formation in mammals or birds. An antibody need not have any enzymatic activity, but we shall assume that it is in some way related to certain degradative enzymes E_j, present in the mammalian cell, and that the rate of formation of an antibody A_j is under the control of the repressor which is specific for the related enzyme E_j.

Whether an enzyme makes its appearance in cellular differentiation, provoked by an organizer, or whether a specific antibody is formed in response to the injection of an antigen, in either case we may assume that the event is triggered by a transient reduction of the concentration of a specific repressor.

If antibodies are paraconstitutive proteins, then, once the concentration of a particular antibody is raised to a sufficiently high level in a lymphatic cell, that cell and all its daughter cells may maintain a high rate of production of that antibody. This would then represent the kind of memory which may form the basis of the so-called secondary response.

The Enzyme-Forming System of the Mammalian Cell.—We assume that the enzyme-forming system in the mammalian cell is described by the same equations which we have postulated for the bacteria. There are certain differences, however, between the mammalian cells here discussed and the bacterial cells. Under physiological conditions bacterial cells as a rule proliferate. Accordingly, in bacteria there is no need for protein turnover, nor do we have any reason to assume that the R moieties of the repressors are hydrolyzed in bacteria at an appreciable rate. In these circumstances, in bacterial cells ρ_0, the total concentration of a particular repressor in the cell, and z^*, the concentration attained by an enzyme which is produced at the full rate, are both determined by the rate of two processes, i.e., the rate at which these entities are formed and the rate at which these entities are diluted through the growth of the bacteria.

In the case of mammalian cells which are not in a state of rapid proliferation, we may in general assume that ρ_0, the concentration of the repressor, and z^*, the concentration of an enzyme which is produced at the full rate, are also determined by the rate of two processes, i.e. the rate at which these entities are produced and the rate at which they are hydrolyzed.

The rate of hydrolysis of the proteins is presumably determined by the level of activity of the proteolytic enzymes in the cell. The rate of hydrolysis of the repressors is presumably determined by the level of activity of some hydrolytic enzyme H which may universally hydrolyze the R moieties of all of the repressors.

We shall assume that the R moiety of the various specific repressors is hydrolyzed in the cell at the same rate whether the specific repressor is free or is combined with the controlling site of an "unattached" enzyme molecule. This is not an unreasonable assumption because the repressor molecule combines with an unattached enzyme molecule in large part by virtue of the chemical affinity of the repressor's M moiety to the controlling site of the enzyme molecule. If the R moiety is indeed a polynucleotide, then the first, or the first few, bases of the polynucleotide may also combine with the controlling site of an unattached enzyme molecule, but the rest of the nucleotide will presumably remain freely exposed to the hydrolytic action of our hypothetical enzyme H.

We shall presently show that if the relevant constants fall within a certain range, quoted below, then according to equations (1) and (5) the enzyme concentration z exhibits the characteristics which we have attributed to paraconstitutive enzymes.

If we equate z given by (1B) and given by (5A), we obtain a cubic equation for ρ. For the below-quoted range of the constants this cubic equation has three positive roots which represent the self-sustaining free repressor concentrations. Accordingly, there may be three self-sustaining pairs of values for ρ and z which we shall designate (in the order of increasing values of z and decreasing values of ρ) with ρ_1, z_1, ρ_2, z_2, and ρ_3, z_3.

Of these, three self-sustaining pairs of values the first and the last pair, ρ_1, z_1 and

ρ_3, z_3 represent stable points. The intermediate pair ρ_2, z_2 represents an unstable point.

We shall refer to z_3 as a high stable enzyme concentration and to ρ_1 as the high stable repressor concentration. To ρ_2 we shall refer as the "critical" repressor concentration.

In the case of a paraconstitutive enzyme, if ρ, the free repressor concentration, is lowered below ρ_2, the critical free repressor concentration (for a period of time which is sufficiently long for the establishment of a stationary state), and if thereafter the free repressor concentration is permitted to find its own level, then the free repressor concentration will decrease to its low stable value ρ_3. Correspondingly, the enzyme concentration will rise to its high stable value z_3.

For any arbitrarily chosen value of ρ which we maintain in the cell long enough to establish a stationary state, we can determine from equations (1B) and (5A) whether the free repressor concentration would thereafter fall or rise in the cell when it is allowed to find its own level. In order to make this determination we substitute the arbitrarily chosen value ρ (to which we shall refer as the test value) into (1B) and (5A), and find from both of these expressions the corresponding value for z.

The rate of enzyme production, for a given free repressor concentration, is expressed by (5A) and if the value for z obtained from this expression is higher than the value for z obtained from (1B), then we may say that the repressor concentration will decrease when it is permitted to find its own level. In the opposite case we may say that the repressor concentration will increase when it is permitted to find its own level.

We propose to utilize below this simple rule, in order to establish the fact that there exist two stable self-sustaining values ρ_1 and ρ_3, if the constants fall within the range quoted below.

We shall now show that if we have

$$\frac{z^*}{K(\lambda - 1)} > 3 \tag{9}$$

and

$$\rho_0/z^* < 10 \tag{10}$$

then there is a stable point at which we have for the low self-sustaining free repressor concentration ρ_3

$$0 < \rho_3 < \rho_0/\lambda.$$

In order to show this we choose for our test concentration $\rho = \rho_0/\lambda$. Substituting this value of ρ into (5B) we obtain

$$\frac{z(\text{from 5B})}{z^*} = \frac{1}{2}$$

Substituting the same value for ρ into (1A) we obtain

$$\frac{z(\text{from 1A})}{z^*} = \frac{\rho_0}{\lambda}(1 + \lambda) + \frac{K(\lambda - 1)}{z^*}.$$

If we take (9) and (10) into account we see that the value for z (from 1A) is less than the value for z (from 5B). Therefore, ρ, when it is permitted to find its own level (after being held at $\rho = \rho_0/\lambda$ for an adequate period of time), will decrease and move to its low stable value, ρ_3.

For the corresponding high stable value of the enzyme concentration z_3 we may write

$$z^*/2 < z_3 < z^*.$$

Next, we propose to determine ρ_1, the high stable concentration of the free repressor, and ρ_2, the critical concentration of the free repressor. We obtain these self-sustaining values from (1B) and (5A) by writing

$$z \text{ (from 1B)} = z \text{ (from 5A)} \tag{11}$$

and by finding the roots of this equation.

Writing out (11) explicitly we obtain

$$\rho_0\left(1 - \frac{\rho}{\rho_0}\right) + K\left(\frac{\rho_0}{\rho} - 1\right) = z^* \frac{1}{1 + \lambda\rho/\rho_0} \tag{11A}$$

which we may also write in the form

$$\lambda = \frac{1}{\rho_0/z^*(1 - \rho/\rho_0)\rho/\rho_0 + K/z^* - K/z^*(\rho/\rho_0)} - \frac{1}{\rho/\rho_0}. \tag{11B}$$

If we have

$$\frac{\rho}{\rho_0}\frac{\rho_0}{z^*} \gg \frac{K}{z^*} \tag{12}$$

and

$$\frac{z^*}{\rho_0}\frac{1}{1 - \rho/\rho_0} \gg 1, \tag{13}$$

then we may simplify (11B) by leaving off the second term and we thus obtain

$$\lambda = \frac{1}{\rho_0/z^*(1 - \rho/\rho_0)\rho/\rho_0 + K/z^* - K/z^*(\rho/\rho_0)} \tag{14}$$

or

$$(\rho/\rho_0)^2 - (1 - K/\rho_0)\rho/\rho_0 + z^*/\rho_0\lambda - K/\rho_0 = 0 \tag{14A}$$

or

$$\frac{\rho_1}{\rho_0} = \frac{1 - K/\rho_0 + \left|\sqrt{[1 - K/\rho_0]^2 - 4[z^*/\rho_0\lambda - K/\rho_0]}\right|}{2} \tag{14B}$$

and

$$\frac{\rho_2}{\rho_0} = \frac{1 - K/\rho_0 - \left|\sqrt{(1 - K/\rho_0)^2 - 4[z^*/\rho_0\lambda - K/\rho_0])}\right|}{2}. \tag{14C}$$

The quantity under the square root in (14B) and (14C) is positive if we have

$$(1 - K/\rho_0)^2 > 4/5 \tag{15}$$

and

$$\lambda > 5z^*/\rho_0. \tag{16}$$

We shall now, for the purposes of our discussion, assume a set of constants which satisfy the inequalities that we have assumed above and actually compute the ρ_1/ρ_0 and ρ_2/ρ_0 from (14B) and (14C) in order to obtain the values for ρ_1 and ρ_2.

For the purpose of our discussion we assume the following set of constants:

$$z^* = 10^{-3} \text{ mol/l}; \ \rho_0 = 10^{-8} \text{ mol/l}; \ K = 10^{-10} \text{ mol/l};$$
$$K^* = 10^{-14} \text{ mol/l}.$$

This set of values satisfies the inequalities assumed above (which represent a sufficient condition for an enzyme to be paraconstitutive) and, in particular, we obtain for this set of constants $\lambda = \rho_0/K^* = 10^6$ and $z^*/K(\lambda - 1) \approx 10$.

The following comment may be made concerning the particular values chosen for our constants:

$x^* = 10^{-3}$ mol/l means that if, even at zero repressor concentrations, the enzyme were formed at the rate corresponding to (5A), then the amount of enzyme in the cell would just about equal the total protein content of the cell.

$K = 10^{-10}$ mol/l corresponds to a free energy change of $\Delta F = 14{,}000$ cal/mol for the combination of a repressor molecule with the controlling site of an "unattached" enzyme molecule. It seems likely that both the metabolite moiety and the R moiety of the repressor would need to have a substantial chemical affinity to the controlling site of the unattached enzyme molecule in order to have a free energy change of this magnitude.

For the above quoted values of the constants we obtain from (14B) for the high stable repressor concentration $\rho_1 \approx 0.9\ \rho_0$ and from (14C) for the critical repressor concentration $\rho_2 \approx 0.11\ \rho_0$.

This means that it would be sufficient to lower the free repressor concentration to about one tenth of ρ_0 in order to trigger the enzyme-forming system and cause the cell thereafter to maintain, indefinitely, the enzyme at a high concentration, i.e. at a concentration lying somewhere between $z^*/2$ and z^*.

The set of values which we have assumed for our constants was selected because it appears conceivable that a quite similar set of values might hold for antibodies formed in the lymphatic cells of the adult rabbit that are capable of forming antibodies which are specific for an antigen injected into the rabbit.

These cells might be characterized—in comparison to the lymphatic cells of the newborn rabbit and the non-lymphatic somatic cells of the adult rabbit—by an increased level of activity of the hypothetical hydrolytic enzyme H. On this basis, we may assume that the value of ρ_0 and of the repression factor λ that holds for the lymphatic cells of the adult rabbit which are capable of forming antibodies is perhaps ten times lower than the corresponding values in those other cells, which are not capable of forming antibodies.

This possibility is discussed in detail in the following paper, "The molecular basis of antibody formation," which attempts to explain a number of phenomena in-

volved in antibody formation on the basis of the phenomenon of enzyme repression in bacteria.

It is conceivable that in attempting to build a theory of antibody formation on this foundation we may be building a house of cards, for in spite of the rapid progress made in the last two years with respect to enzyme repression in bacteria, many of the conclusions drawn from the experiments are still largely based on circumstantial evidence. This holds in particular for the conclusion, here adopted, that the repressor controls the rate at which the enzyme is formed by the enzyme-forming site rather than the rate of formation of the enzyme-forming site itself.

Clearly, we cannot attempt at present to say how antibodies are actually formed; at best we may be able to say how antibodies might conceivably be formed. But to be able to say even this much might be of some value.

I had the privilege of discussing the thoughts expressed in this paper with Dr. Maurice S. Fox, The Rockefeller Institute, New York, and it is a pleasure to acknowledge his help in clarifying the issues involved.

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† A similar model was proposed independently by O. Maaløe and presented at an informal seminar at the Cavendish Laboratory in Cambridge in 1958; it will be described in *Microbial Genetics* (Cambridge University Press, 1960).

‡ Notations:

REP	is the specific repressor which controls the rate of formation of an enzyme and which is composed of two moieties, an M moiety and an R moiety.
M	is a metabolite, which forms the metabolite moiety of the repressor.
R	is the R moiety of a repressor, which is specific for enzymes lying along a certain stretch of a given biochemical pathway.
K^*	is the equilibrium constant for the dissociation of a repressor molecule from the controlling site of the corresponding enzyme molecule that is attached to its enzyme-forming site.
K	is the equilibrium constant for the dissociation of a repressor molecule from the controlling site of the "unattached" enzyme molecule, present in the cell.
$K^*_{\bar{M}}$	is the equilibrium constant for the dissociation of a chemical analogue $\bar{M}$, of the metabolite M, from the controlling site of an enzyme molecule attached to its enzyme-forming site.
$K_{\bar{M}}$	is the equilibrium constant for the dissociation of the chemical analogue $\bar{M}$ from the controlling site of an unattached enzyme molecule contained in the cell.
ρ_0	is the concentration of the molecules of the repressor REP in the cell.
ρ	is the concentration of the free repressor molecules in the cell, i.e. of those repressor molecules which are not combined with an unattached enzyme molecule.
μ	is a concentration of the chemical metabolite $\bar{M}$ in the cell.
z	is the concentration of the "unattached" enzyme molecules in the cell.
z^*	is the concentration that the enzyme would attain in the cell, for $\rho = 0$, if we disregard the fact that factors, which our formulae do not take into account, may limit the rate of the formation of the enzyme for high values of z.
λ	is the repression factor defined by $\lambda = \rho_0/k^*$.

*THE MOLECULAR BASIS OF ANTIBODY FORMATION**

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In a preceding paper we have discussed the phenomenon of enzyme repression in bacteria and presented a model for a kind of cellular differentiation which might conceivably play a role in embryonic development. In the present paper we propose to discuss the molecular basis of antibody formation in the light of this model.

The Experimental Facts.—We shall list in the following the major immunological phenomena that we may regard as well established. Throughout this paper we shall restrict our discussion to one animal, the rabbit, and to one class of antigens, soluble proteins.

(1) When a soluble protein antigen is injected into an adult rabbit for the first time, the rabbit responds after about 5 days with the formation of antibodies which are specific for this antigen. Any remaining antigen will be rapidly eliminated from the circulation, and for a while there will be a substantial concentration of antibody present in the circulation. Subsequently the concentration of the antibody in the circulation will gradually decrease and after a while it may become no longer detectable. This is the so-called primary response.

(2) If, a few weeks after we have injected into the rabbit a soluble antigen, such as a foreign serum protein, we inject the same antigen again, then three days later the rabbit may respond with a copious production of antibody and the antigen will be rapidly eliminated from the circulation. The concentration of the antibody in the circulation may reach a high level, then fall off rather slowly and remain appreciable for a long time. This is the secondary response.

In response to the same amount of antigen injected, a much larger amount of antibody may be produced in the secondary response than in the primary response.

The secondary response can be elicited even a very long time after the antibody has disappeared from the circulation, subsequent to the first injection of the antigen. The readiness of the rabbit to exhibit such a secondary response represents some sort of a memory which fades away only very slowly.

(3) Albert Coons has found in the rabbit that if one evokes the secondary response in the manner described above, then, after 48 hours, clusters of cells which contain antibodies specific for the antigen may be found in the lymph nodes which are involved. The clusters found 4 days or 8 days after the injection are on the average larger than the clusters found after 48 hours. The cells which compose these clusters are small and round and have the appearance of plasma cells. Mitotic figures can be seen in the clusters, indicating that there is proliferation of the cells producing the antibody. The clusters vary in size; the larger ones consist of about a hundred or perhaps a few hundred cells.

(4) For a few days after birth, the rabbit is not capable of forming antibody in response to the injection of an antigen. If a newborn rabbit is injected with a *large* quantity of a soluble protein antigen, then later on when this rabbit becomes an adult and is capable of forming antibodies in general, it will still remain incapable of forming antibody against the antigen which was administered to it immedi-

ately after birth, even though by that time the concentration of the antigen in the circulation may have fallen to a very low level. This is the phenomenon of enduring immune tolerance.

(5) If an adult rabbit is given an X-ray dose of about 400 r to its whole body and a few days later an antigen is injected into the rabbit, then the rabbit will not form antibodies against this antigen. After a while the effect of the X-ray exposure will wear off and the rabbit is then again capable of forming antibodies.

If after the X-ray exposure, at the time when the rabbit is not capable of responding with the formation of antibody, a *large* quantity of an antigen, such as a foreign serum protein, is injected into the rabbit, then the rabbit will remain incapable of forming antibodies against this particular antigen, even after the effect of the X-ray exposure has worn off and the concentration of the antigen in the circulation has fallen to a level which is no longer detectable. Thus the X-rayed adult rabbit exhibits the phenomenon of enduring immune tolerance.

(6) If the antigen is injected into the rabbit prior to the exposure to the X-ray dose, then this exposure will not prevent the rabbit from forming antibodies to the antigen.

(7) If a rabbit has been pre-immunized with an antigen, then exposure of the rabbit to an X-ray dose of 400 r a few days prior to the injection of the antigen will not block the secondary response, i.e. in such a rabbit the injection of the antigen will evoke the secondary response even though the injection is given a few days following the administration of the X-ray dose.

The Enzymes of the Lymphatic Cells.—We postulate that, in general, the somatic cells of the rabbit contain a number of diverse biochemical pathways, j, $j+1$ $j+2$, etc., and that a number of enzymes lie along each such pathway. This might be represented symbolically by writing

$$M \begin{cases} M_j^1 \xrightarrow{E_j^1} M_j^2 \xrightarrow{E_j^2} M_j^3 \xrightarrow{E_j^3} \cdots\cdots M_j^k \xrightarrow{E_j^k} M_j^{k+1} \\ M_{j+1}^1 \xrightarrow{E_{j+1}^1} M_{j+1}^2 \xrightarrow{E_{j+1}^2} M_{j+1}^3 \xrightarrow{E_{j+1}^3} \cdots\cdots M_{j+1}^k \xrightarrow{E_{j+1}^k} M_{j+1}^{k+1} \\ M_{j+2}^1 \xrightarrow{E_{j+2}^1} M_{j+2}^2 \xrightarrow{E_{j+2}^2} M_{j+2}^3 \xrightarrow{E_{j+2}^3} \cdots\cdots M_{j+2}^k \xrightarrow{E_{j+2}^k} M_{j+2}^{k+1} \end{cases}$$

As this scheme indicates, the metabolites M_j; M_{j+1}; M_{j+2}; etc. of several of these stray biochemical pathways might have a common precursor M.

Let us now consider one of these enzymes, for instance E_j^k. We assume that this enzyme is under the control of the repressor

$$REP_j^k = \{M_j^k - R_j\}$$

We further assume that there is a coupling enzyme C_j^k present in the cell which joins the R moiety of the repressor, R_j, to the M moiety of the repressor, M_j^k. Symbolically this might be indicated by writing

$$M_j^k + R_j \xrightarrow{C_j^k} \{M_j^k - R_j\}.$$

We shall assume in our discussion that the coupling enzymes C are not under the control of any of the repressors here considered.

We shall further assume that both the catalytic site and the controlling site of all these degradative enzymes, E_j, can specifically combine with M_j, the substrate of the enzyme. (See preceding paper.)

The Genes G and the Antibodies A.*—We shall designate by G_j the genes which determine the identity of these degradative enzymes E_j, and we postulate that for each enzyme E_j there is only one corresponding gene G_j contained in each of the two haploid sets of chromosomes. The total number of genes of this category might perhaps be of the order of 10,000 per haploid chromosomal set.

One might be tempted at this point to assume that the enzymes E are themselves the antibodies. We prefer, however, to postulate the following: the genes G have a tendency to get doubled and in the course of evolution each gene G_j may have doubled many times. We assume that when a gene G undergoes doubling, the genes G^* which are formed will in general lack a part, presumably a small part, of the gene G. We assume in particular that the genes G^* differ from the genes G in two respects, which are as follows:

(a) the genes G^* lack the tendency of the genes G to undergo doubling;
(b) the proteins which are determined by the genes G_j^* resemble closely the enzyme E_j which is determined by the corresponding gene G_j but they may lack the catalytic activity of the enzyme. *We postulate that these proteins are the antibodies A_j.*

The controlling site of an antibody A_j has the same specific combining affinity to the metabolite M_j as has the controlling site of the enzyme E_j. Also the catalytic site of an antibody A_j may have the same specific combining capacity for the metabolite M_j as the catalytic site of enzyme E_j, to which it is related, *and if this is the case then we are dealing with a divalent antibody.*

The spontaneously occurring doubling of the genes G might be balanced by spontaneously occurring deletions of the genes G^*, and an equilibrium might be maintained on this basis, in the absence of genetic deaths. In such an equilibrium there might be present a number of genes G^* corresponding to each gene G. However, deletions of the genes G and their mutations to incompetence *would* have to be balanced by the occurrence of genetic deaths, in a state of mutational equilibrium.

There is a limit to the amount of genetic deaths which we may assume to occur per generation, because no species of mammals could remain in existence if the amount of genetic deaths were too high. This consideration does not permit us, however, to set an upper limit for the number of genes G^* present per gene G, because we are assuming that with respect to the genes G^* mutational equilibrium is maintained in the absence of genetic deaths.

We may set an upper limit for the total number of genes G^* on the basis of the amount of *DNA* present in the cell, but this comes out to be very high; *if we assume that the weight of the mammalian gene is about the same as the weight of the bacterial gene, then the amount of DNA in the mammalian cell would be sufficient to account for one million genes.*

The Nature of the Antigen.—For the purpose of our discussion an antigen P_j may be represented as a molecule which is composed of a non-antigenic protein molecule P_0 to which are coupled m identical groups $\bar{M}_j$ (to which we shall refer as a hapten). Accordingly, we may represent such an antigen P_j symbolically, by writing

$$P_j = \{P_0 - (\bar{M}_j)_m\}$$

One can prepare rather simple artificial antigens of this type by diazo-coupling

a hapten, some small molecule, arsanylic acid for instance, to a protein which is not antigenic in the rabbit. If m, the average number of such haptens per protein molecule, is made large enough ($m \geq 10$) we may have a good antigen which will elicit the formation of antibodies specific for the hapten.

If the hapten $\bar{M}_j$ is a chemical analogue of the metabolite M_j, then the antibody molecule A_j is capable of combining with the antigen molecule P_j by virtue of the specific chemical affinity of the catalytic site and of the controlling site of the antibody molecule A_j to the hapten $\bar{M}_j$.

In the case of a natural protein antigen, such as a foreign serum protein, we have a more complicated situation because one protein molecule might carry a certain number, m_1, of one kind of determinant group and also a certain number, m_2, of another kind of determinant group, etc. To these determinant groups we shall, for the sake of brevity, also refer as haptens.

More complex artificial antigens may be prepared by coupling a hapten to a natural protein which is itself antigenic in the rabbit.

The Rabbit Antibody-Antigen System.—If antibody which is obtained from the rabbit is mixed with the antigen for which it is specific, then within certain concentration limits a precipitate may be formed in which each antigen molecule may be combined with a number of antibody molecules, and each antibody molecule may be combined with two antigen molecules. The concentration limits within which such a precipitate is formed define the so-called equivalence zone where the supernatant over the precipitate contains only small quantities of both the antigen and the antibody. The free antigen concentration is exceedingly low in equilibrium with such a precipitate.

A precipitate may also be formed if the rabbit antibody is present in large excess. Such a precipitate may be represented as consisting of units in which a number of antibody molecules (the number depending on the size of the antigen molecule) are combined with one antigen molecule; the precipitate forms because such units stick to each other. The free antigen concentration is low in equilibrium with a precipitate of this type also.

No antigen-antibody precipitate is formed if antigen is present in great excess. Presumably in this case each divalent antibody molecule is combined with two antigen molecules. One of the antigen molecules is combined with the antibody molecule at the controlling site, and the other is combined with it at the catalytic site. Each antigen molecule is combined, however, with one antibody molecule only.

Proteins which may have a tendency to stick to gamma globulins may be co-precipitated when an antigen antibody precipitate is formed in their presence. An example for proteins which may be co-precipitated in this manner are the serum "complements"; which are "fixed" when an antigen-antibody precipitate is formed in their presence.

The Coupling Enzyme-Antigen System.—If the hapten $\overline{M}_j$ of the antigen P_j is a chemical analogue of the metabolite M_j, then the antigen can reversibly combine with the coupling enzyme C_j by virtue of the specific chemical affinity of the hapten to a part of the catalytic site of the coupling enzyme.

We postulate that the coupling enzymes resemble those antibodies of the rabbit which form a precipitate with antigen when the antibody is in excess. Accordingly, at low antigen concentrations a number of coupling enzyme molecules might be

combined with one antigen molecule and such units may then stick to each other and form a precipitate. We may assume that the free antigen concentration can be very low in equilibrium with such a precipitate.

The "Sensitive" Lymphatic Cells.—We assume that the cells of the lymphatic system go through a maturation process that carries the cell from its initial form, presumably the stem cell, to its mature form, presumably the plasma cell. When a lymphatic cell reaches a certain phase in this maturation process it becomes "sensitive" in the sense that it becomes capable of responding to the exposure to a soluble antigen with the formation of a specific antibody. We assume that what renders the cell sensitive, when it reaches this phase of its maturation process, is a general lowering of the repression factors to a value of perhaps $\lambda = 10^6$. (For definition of the repression factor see preceding paper.) This might be brought about by an increase in the activity of an enzyme which can universally hydrolize the R moieties of the repressors. In the preceding paper we have postulated the existence of such an enzyme and designated this hypothetical enzyme as the "hydrolase."

The set of constants assumed in the preceding paper, for the enzyme-forming system of the mammalian cell, we assume to hold for the antibody-forming system of the "sensitive" lymphatic cells. In the immature form of the lymphatic cells and in the rest of the somatic cells the concentration of the repressors ρ_0 and the corresponding repression factors λ presumably have a much higher value.

The Primary Response.—We assume that when a soluble antigen is injected intravenously or intraperitoneally into the rabbit, it penetrates into the cytoplasm of all the lymphatic cells. We further assume that if the antigen stays in the circulation long enough, the free antigen concentration in the lymphatic cells will be the same as the free antigen concentration in the circulation. This means that the chemical potential of the antigen inside the cytoplasm of the lymphatic cell is the same as the chemical potential of the antigen in the circulation.

According to the views here adopted, the primary response comes about in the following manner: when a soluble antigen P_j is injected into the rabbit it will diffuse into the lymphatic cells and precipitate the coupling enzyme C_j. As the result of this, the rate at which the repressor REP_j is formed will be reduced, and the concentration of this repressor will begin to fall. As the concentration of the repressor falls, the rate of formation of the antibodies A_j will increase.

Antibody molecules A_j present in the cell bind molecules of the repressor REP_j because of the specific affinity of the repressor molecule to their controlling site. Accordingly, in a sensitive cell, as the concentration of the antibody molecules A_j rises and the concentration of the repressor molecules REP_j falls, at some point in time the enzyme-forming system will lock and from then on the cell will produce the antibody molecules A_j at a high rate, even in the absence of antigen.

This sustained antibody production, by the cells in which the enzyme-forming system has locked in the primary response, represents a kind of memory, and it may account for the sustained immunity manifested by the rabbit.

Possibly a cell might lock simultaneously for the production of antibodies specific for two different haptens. But once a cell has locked for the production of an antibody, which is thereafter produced at a high rate, then subsequently a new stationary state establishes itself in the cell. The cell is then no longer "sensitive,"

so that if it is exposed to another antigen it will not lock for the production of the corresponding antibody.

The Complexities of the Primary Response.—When the primary response is elicited in the rabbit by injecting an antigen which consists of a non-antigenic protein P_0 to which are coupled m haptens $\bar{M}_j$, there will combine with an antigen molecule not only molecules of the coupling enzymes C_j but also molecules of the enzyme E_j and of the antibodies A_j.

The phenomena accompanying the primary response are even more complicated if the artificial hapten is coupled not to a protein which is non-antigenic in the rabbit but, for instance, to a foreign serum protein which *is* antigenic in the rabbit. When such an antigen diffuses into the lymphatic cells, various coupling enzymes corresponding to the various determinant groups carried by the antigen molecule, and also the corresponding enzymes E and antibodies A may combine with the antigen and form a precipitate.

On this basis it is possible to explain why a conjugated protein carrying an artificial hapten elicits more antibody directed against the artificial hapten if the protein is a foreign protein which is a good antigen in the rabbit, rather than if it is one of the rabbit's own serum proteins.

The Secondary Response.—We shall postulate that the cells of the lymphatic system contain a hypothetical enzyme S which, when present at high concentration, will inhibit cell division. We shall further postulate that this hypothetical enzyme S resembles complement, inasmuch as it can be co-precipitated if an antigen-antibody precipitate is formed in its presence.

When an antigen P_j is injected into a rabbit for the first time, a certain number of the sensitive lymphatic cells will lock and henceforth produce the antibodies A_j at a high rate. When a lymphatic cell locks and produces such proteins at a high rate, it will from then on produce most other proteins, including the hypothetical enzyme S, at a low rate. Thus, following the locking of the lymphatic cell in the primary response, the concentration of the enzyme S will fall, and it may reach a new stationary level within a few days or weeks.

If, about four weeks after the first injection of the antigen P_j, the same antigen is injected into the rabbit again, it will diffuse into the lymphatic cells and it will form a precipitate with the antibodies A_j in those lymphatic cells which have locked at the time of the first injection of the antigen. By the process of co-precipitation a certain quantity of the enzyme S will thereby be fixed, and, inasmuch as at the time of the second injection the level of this enzyme in the locked cells is low, the concentration of the enzyme may fall to the point where it can no longer inhibit cell division.

Thus, the second injection of the antigen will lead to proliferation of those cells which have locked at the time of the first injection of the antigen. This is our explanation of the secondary response.

If the views here adopted are correct, we may expect that the secondary response would be elicited by any compound carrying a hapten, which forms a precipitate with the antibody that is directed against this hapten, whether or not the compound is capable of eliciting the primary response.

The finding of compounds which are weak antigens, in the sense that they will

elicit only a weak primary response, but are good antigens, in the sense that they will elicit the full secondary response, would lend support to our theory.

A conjugated protein, obtained by coupling an artificial hapten to rabbit serum albumin, is supposed to be a very weak antigen in the rabbit, in the sense that it does not elicit in the primary response the formation of an appreciable quantity of antibody directed against the artificial hapten. On the other hand, a conjugated protein obtained by coupling an artificial hapten to a foreign serum globulin, which is antigenic in the rabbit, is supposed to be a very good antigen in the sense that it will elicit in the primary response the formation of a substantial quantity of antibody directed against the artificial hapten. (Oral communication, Herbert Anker, 1959.)

On this basis, our theory predicts that if we pre-immunize the rabbit with a conjugated foreign serum globulin and evoke the secondary response with the conjugated rabbit serum albumin, we should obtain in the secondary response a substantial quantity of antibody directed against the hapten. If, however, we pre-immunize the rabbit with the conjugated rabbit serum albumin and evoke the secondary response with the conjugated foreign serum globulin, then we should obtain in the secondary response a less substantial production of the antibody directed against the hapten.

In interpreting the results of an experiment of this type, one must keep in mind that when an antigen is injected for the second time one obtains not only a secondary response but also a "primary response," in which sensitive lymphatic cells will lock for the formation of the specific antibody. It should be possible, however, to distinguish these two responses, because the release of the antibody into the circulation from the secondary response presumably precedes its release from the "primary response."

The Decay of the Primary Response.—In the primary response the release of antibody into the circulation does not persist long at a substantial rate after the antigen has been eliminated from the circulation. It is conceivable, though by no means certain, that, as far as the release of antibody into the circulation is concerned, the phenomena which characterize the secondary response may play a part in the primary response also.

When a cell locks for the production of an antibody in the primary response the concentration of the antigen present in the circulation might be high enough to lead to the formation of an antibody-antigen precipitate in the locked cell and to a co-precipitation of the enzyme *S*. This might cause a proliferation of the locked cell as described above, in connection with the secondary response.

The locked cells which divide may disintegrate as fast as they are produced and release their antibody content into the circulation.

Soon after the antigen disappears from the circulation, this enforced proliferation of the locked cells may cease, but we may assume that the locked cells will keep on dividing, even though rather slowly, and releasing their protein content into the circulation. The amount of antibody produced which is specific for any given antigen may not be appreciable, but the total gamma globulin production of the adult animal may be a measure of the rate at which the locked lymphatic cells divide and disintegrate.

Newborn rabbits are not capable of forming antibodies and we may perhaps

assume that in the lymphatic cells of the newborn rabbit hydrolase activity is low, and therefore the repression factors λ are high. On this basis we may understand why lymphatic cells of the newborn rabbit cannot lock for the production of an antibody when they are exposed to an antigen.

The fact that in the young rabbit, just as in many other young animals, the rate of production of gamma globulin is low supports the view here adopted that the gamma globulins are produced by the locked lymphatic cells. The lymphatic cells which have not locked might not divide at all or might divide exceedingly slowly.

Immune Tolerance Induced in the Newborn Rabbit.—If a large amount of an antigen P_j is injected into a newborn rabbit, which cannot form antibodies, the antigen will diffuse into the lymphatic cells and there will be a condition of antigen excess both with respect to the coupling enzymes C_j and the antibodies A_j.

At the time, perhaps two weeks after birth, when some of the lymphatic cells of the rabbit become "sensitive" and capable of forming antibodies, the antigen concentration in the circulation will still be high.

The presence of a high concentration of the antigen P_j will prevent the locking of these cells with respect to the production of the antibodies A_j, because the haptens $\bar{M}_j$ of the antigen P_j compete with the repressor REP_j for the controlling site of the antibodies A_j.

The concentration of the antigen in the circulation of the rabbit will slowly fall and after several months it might reach a very low value. Nevertheless, the rabbit may still exhibit at that time specific immune tolerance with respect to the antigen. We may attempt to account for this phenomenon as follows:

If an antigen molecule is combined with the controlling site of the "attached" enzyme molecule we may assume that it will set up a steric hindrance and repress the formation of the antibody molecule in much the same way as would the specific repressor molecule.

We shall designate the enzyme-forming site which is specific for the formation of an antibody A_j as the ribosome B_j*. For the purpose of our discussion we may assume that corresponding to each gene G_j* which determines an antibody A_j there is present in the cell one and just one ribosome B_j*. Since we have assumed that there are a number of genes G_j* which correspond to the gene G_j, there will also be a number of ribosomes B_j* for each gene G_j. (The antibodies A_j which are made by the different ribosomes B_j* may differ somewhat from each other in their specific combining capacity with respect to the metabolite M_j and the hapten $\bar{M}_j$.)

We shall assume that each antibody A_j is formed inside the corresponding ribosome B_j*, and that the ribosome is a semi-closed structure.

We assume that water, salts, and small protein molecules, including the hypothetical enzyme U postulated in the preceding paper, may all diffuse freely in and out of the ribosome. The antibody molecule A_j which is produced in the ribosome can, however, not diffuse out of the ribosome, and its concentration will therefore rise until the osmotic pressure inside the ribosome exceeds the osmotic pressure in the cytoplasm outside of the ribosome. At that point water will begin to diffuse into the ribosome and as the hydrostatic pressure inside the ribosome increases, the ribosome may open up sufficiently to permit escape of a few antibody molecules. On the basis of such a model we may expect that even in cells which are not locked

for the production of an antibody each ribosome contains the antibody, for which it is specific, at a high concentration.

The concentration of the antigen injected into the newborn rabbit will remain high in the circulation for an extended period of time, and during this time enough antigen P_j will diffuse into the ribosome B_j^* to form an antigen-antibody precipitate. The "attached" antibody molecule A_j in the ribosome may be combined at its controlling site with an antigen molecule and may form part of the precipitate. Because the concentration of the free antigen in equilibrium with such a precipitate is low, the precipitate may still persist in the ribosome at the time when the concentration of the free antigen in the circulation has fallen so low as to be undetectable. In this manner we may account for enduring immune tolerance.

Accordingly, enduring immune tolerance requires the presence of the antigen in the lymphatic cells, but does not require the antigen to be present in the circulation at a detectable concentration.

Specific immune tolerance could not endure for long if the lymphatic cells of the rabbit which have *not* locked for the production of any antibody were to divide at a substantial rate, because new ribosomes B_j^* are presumably formed by the genes G_j^* when the cell divides, and antibody production in these new ribosomes would not be blocked. (Cells which have locked for the production of an antibody may be assumed to undergo divisions at an appreciable rate, but these cells are not capable of responding to the exposure of an antigen with the formation of the specific antibody, and would therefore not abolish immune tolerance.)

The Case of the X-Rayed Rabbit.—We may attribute the inability of the rabbit to respond with the formation of antibody, if the antigen is injected several days after the rabbit has been exposed to an X-ray dose of 400 r, to the inability of its lymphatic cells to lock for the formation of an antibody.

On this basis we may expect that, if a large quantity of an antigen is injected into the rabbit, several days after the rabbit has been exposed to an X-ray dose of 400 r the rabbit will exhibit enduring immune tolerance with respect to this antigen.

If an X-ray dose of 400 r is administered to the rabbit after the injection of an antigen, then by the time the X-ray exposure takes effect many of the lymphatic cells of the rabbit will have locked for the production of antibody directed against the antigen. These locked cells will proceed to form antibody, unaffected by the X-ray exposure. Accordingly, in this case the X-ray exposure will not prevent the rabbit from responding to the injection of the antigen with the formation of antibody.

Since the antibody released into the circulation in the secondary response is released by cells that have locked for the formation of the antibody at the time of the primary response, or by their descendants, there is no reason to expect that exposing the pre-immunized rabbit to an X-ray dose of about 400 r should inhibit the secondary response.

Concluding Remarks.—If the model for cellular differentiation, presented in the preceding paper, should in fact correctly describe the molecular basis of antibody formation, then we could be rather confident that the above given explanations for the major immunological phenomena are essentially correct. This holds in particular for the role we have attributed to the "locking" of sensitive lymphatic cells in the primary response and for the notion that the primary response and the second-

ary response are basically different phenomena. We cannot be equally confident, however, of the correctness of various assumptions which relate to details and which we introduced for the sake of the concreteness of the discussion. Among these are the assumptions which relate to the mechanisms by which the antigen lowers the repressor concentration and the assumptions which relate to the structure of the ribosomes B^*. As far as such details are concerned it might well be that our assumptions will have to be modified later on, in the light of future experimental data.

In the process of formulating the thoughts expressed in this paper I had the privilege of discussing the subject with Dr. Maurice S. Fox, The Rockefeller Institute, New York; Dr. Howard Green and Dr. Baruj Benacerraf, New York University College of Medicine; and Dr. Herbert Anker, The University of Chicago. It is a pleasure to acknowledge their helpful suggestions and criticism.

* This paper is the continuation of a paper entitled "The Control of the Formation of Specific Proteins in Bacteria and in Animal Cells," pages 277–292 of this issue of these PROCEEDINGS. The concepts, notations, and equations of the first paper carry over to this paper.

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*PURIFICATION OF A NERVE-GROWTH PROMOTING PROTEIN FROM THE MOUSE SALIVARY GLAND AND ITS NEURO-CYTOTOXIC ANTISERUM**

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Introduction.—We have reported the isolation of a protein from snake venom which specifically promotes the growth of sensory and sympathetic nerve cells, both in tissue culture and in the living chick embryo.[1,2,3] The discovery of a similar factor in the salivary glands of the mouse, rat, and hamster,[1] extended the investigation to include mammals.

The first part of this paper is concerned with (*a*) the purification and nature of the nerve-growth factor in the submaxillary gland of the mouse, and (*b*) its effect, when injected into the mouse, on the gross chemical composition of the sympathetic ganglia.

In the course of this study we were able to prepare an antiserum to the purified growth factor isolated from the salivary gland. The antiserum inhibited, in tissue culture, the biological activity of the antigen. This gave us an opportunity to examine a question raised in our previous work concerning the possible physiological role (if any) of the growth factor in the normal organism. If a similar factor operates in the normal mouse, then the injection of the antiserum might interfere with its function. We have indeed found that the subcutaneous injection of this antiserum *in vivo* results in a specific atrophy and destruction of the sympathetic

nerve cells not only of the mouse, but also of the rat, cat, and rabbit. The cytological effects on the nerve cell of both the growth factor and the specific antiserum are described in two communications by Levi-Montalcini and Booker (in this issue of the PROCEEDINGS). These observations suggest, but do not prove, that the growth factor or an antigenically similar molecule is involved in the maintenance of the nerve cells of the sympathetic system.

Apart from this point, the more or less complete elimination of the sympathetic nervous system by a specific antiserum opens up new areas for physiological research.

Materials and Methods.—The submaxillary glands were isolated from adult (over 22 gm body weight) male Swiss mice. The carboxymethyl cellulose (CM-cellulose) and diethylaminoethyl cellulose (DEAE-cellulose) preparations have been described.[3]

The assay for nerve-growth promoting activity was made using sensory ganglia from 8–9 day chick embryos explanted in hanging drop tissue cultures. The amount of fiber outgrowth was observed after 18 hr of incubation. The details of the procedure have been presented elsewhere.[1,3] The assay was sensitive to twofold changes in concentration of the active material; smaller changes were not detectable.

The protein determinations were made using the Folin phenol reagent[4] with bovine albumin as a standard. The total nucleic acid content of the ganglia was estimated by the ultraviolet absorption of a hot acid extract.[5] It was assumed that both DNA and RNA have an ϵ (P) of 9850 at 268.5 mμ. The protein was determined on the residue after the hot acid extraction. The DNA content of ganglia was determined by a fluorometric procedure.[6]

Experimental Results.—Distribution of the growth factor: In a series of experiments the concentration of the factor in the submaxillary gland, obtained from both male and female mice at various ages, was examined. The glands were excised, homogenized in isotonic sodium chloride, and aliquots were assayed for nerve growth promoting activity in tissue culture. The results are shown in Table 1. Up

TABLE 1

NERVE GROWTH FACTOR CONTENT OF THE SUBMAXILLARY GLAND OF MICE DURING MATURATION

Age, Days	Protein Required for a 3+ Response, μg per ml	
	Female	Male
0–17	>8,000	>8,000
25	4,000	1,000
32	1,000	200
41	50	6
44	100	3
50	6	1.5

Aliquots of crude homogenates of the submaxillary gland were assayed for biological activity.

to approximately 17 days of age only traces of activity could be detected. During the period of 25 to 50 days of age, when adult levels are reached, there is a several thousandfold increase in the specific activity of the homogenates. In all age groups the submaxillary gland from male mice showed a higher specific activity than did the corresponding gland of female mice.

Homogenates of the submaxillary gland isolated from adult rats and hamsters

were active, but possessed a specific activity of only $^1/_{1,000}$ of that of the adult male mouse gland. Activity was also detected in the saliva of a mouse injected with pilocarpine to induce salivation. Only traces, if any, could be detected in crude homogenates of the submaxillary gland of the cow at levels up to 5 mg of protein per ml of tissue culture medium.

The sublingual salivary gland of the mouse has regularly shown a specific activity of approximately $^1/_{100}$ of that of the submaxillary gland. In one of three attempts the parotid gland was found to contain a trace of activity.

The presence of the growth factor in certain rodent tumors has been extensively reported.[7-11] Evidence has been obtained that a similar activity may be present in very low concentrations in other normal mouse tissues. Levi-Montalcini *et al.*[9] have reported its presence in the heart muscle of embryonic mice. Traces of activity have occasionally been found in homogenates of the striated muscle of adult mice,[10] and in one of four attempts it has been detected in the blood serum of a normal mouse. Although at levels of 5 mg of protein per ml of tissue culture medium crude homogenates of a variety of other mouse tissues have shown no detectable activity,[1] Bueker[12] has detected some activity in partially purified preparations of thymus, kidney, and muscle. Its presence has recently also been noted in the serum and in the urine of the mouse (R. Levi-Montalcini).

Where examined, the specific activity of tissues other than the salivary gland was never more than $^1/_{5,000}$ of the latter.

It has not been ascertained whether the growth factor is synthesized in the salivary gland or is produced elsewhere and is concentrated in that organ. In any case, there is a striking parallelism between the distribution of the activity as shown in Table 1 and the cytological changes which occur in the salivary gland of the mouse during its post-natal development.[13]

Purification of the growth factor: Adult male mice (22 gm body weight or over) were killed using chloroform. The submaxillary glands were excised and frozen until a sufficient quantity had been accumulated (8 gm wet weight from 70–80 mice). The frozen tissue was homogenized for 2 min with 50 ml of cold distilled water in a Waring blendor. The homogenate was centrifuged for 10 min. The supernatant fluid was decanted and the residue was stirred with 22 ml of distilled water and recentrifuged. The supernatant fluids were combined and the residue discarded. All centrifugations in this procedure were performed at 0–2° at 15,000 $\times$ *g* in an International centrifuge.

To 9 volumes of the combined supernatant fluids was added 1 volume of a stock streptomycin solution. (Stock streptomycin sulfate solution: 0.2 *M*, adjusted to pH 7.8 with NaOH.) The final pH of the mixture was between 6.9 and 7.1. The mixture was allowed to stand in the refrigerator for 3 hr and then was centrifuged for 5 min. The clear red supernatant was retained and the residue (nucleoproteins) was discarded.

Absolute alcohol was then added in a ratio of 0.07 ml per ml of supernatant fluid. The mixture was allowed to stand for 15 min at 0° and was then centrifuged. The small precipitate was discarded. To the supernatant was added absolute alcohol, 0.33 ml for each ml of *original* solution. The mixture was allowed to stand for 45–60 min at −5°, and was then centrifuged for 10 min. The supernatant fluid was discarded. The precipitate contained most of the red pigment and the

growth factor. To the precipitate was added 25 ml of distilled water and the pasty residue was stirred until uniformly dispersed. We found it convenient to freeze and store the material overnight at this stage.

The frozen mixture was then thawed and centrifuged; the precipitate was discarded. The supernatant was fractioned by the addition of a saturated ammonium sulfate solution, pH 7.3. The fraction precipitating between 52.5 and 71 per cent saturation was retained. (A time interval of 15 min at 0° was allowed for the precipitation after each addition of the ammonium sulfate solution.) The active precipitate was dissolved in 25 ml of distilled water to form a clear red solution. The solution was dialyzed, with stirring, for 36 hr against repeated changes of distilled water. The mixture was then centrifuged and the slight precipitate was discarded.

A short column of CM-cellulose was prepared as follows: 1 gm of the powder, in a 2 cm diameter column, was washed with a mixture of 0.5 *M* NaOH, 0.5 *M* NaCl, and then with 0.005 *M* NaCl until the eluate was free of alkali. The dialyzed ammonium sulfate fraction was then passed through the column at a flow rate of 3–4 drops per minute. Under these conditions the active material was not absorbed. The cellulose was washed with 2 column volumes of distilled water and the water clear eluates were combined (CM-1 fraction). All of the red pigment remained adsorbed to the column.

A column of DEAE-cellulose was prepared. 3 gm of the powder, in a 2 cm diameter column, were successively washed with solutions containing (*a*) 0.5 *M* NaOH, 0.5 *M* NaCl, (*b*) 0.1 *M* NaCl, (*c*) 0.1 *M* phosphate buffer, pH 6.0, and (*d*) 0.005 *M* NaCl. The CM-1 fraction was then passed through the column followed by 80 ml of 0.01 *M* NaCl. The eluates were discarded. The active material was then eluted with 70 ml of 0.12 *M* NaCl (DEAE fraction).

A second column of CM-cellulose was prepared. 3.5 gm of the powder, in a 2 cm diameter column, were successively washed with solutions containing (*a*) 0.5 *M* NaOH, 0.5 NaCl, (*b*) 0.01 *M* NaCl, (*c*) 0.1 *M* sodium acetate buffer pH 4.38, and (*d*) 0.01 *M* NaCl. The DEAE fraction was now passed through the column. The column was then washed with 40 ml of 0.1 *M* NaCl and then with 100 ml of 0.3 *M* NaCl. All the eluates thus obtained were discarded. The active material was then eluted with 70 ml of 0.75 *M* NaCl (CM-2 fraction).

A third column of CM-cellulose was prepared as follows: 500 mg of the powder in a 1 cm diameter column was washed as directed above for the CM-2 fractionation, except that the final wash is made with 0.1 *M* NaCl instead of 0.01 *M* NaCl. The CM-2 fraction was dialyzed overnight against 0.1 *M* NaCl and passed through the column. The column was then washed successively with 15 ml of 0.1 *M* NaCl, 15 ml of 0.3 *M* NaCl, and 20 ml of 0.35 *M* NaCl. These eluates were discarded. The active material was then eluted with 15 ml of 0.75 *M* NaCl. This solution is stable at least for several weeks when kept frozen, although a gradual flocculation of the active material occurs.

Typical yields and biological activities at the various fractionation steps are shown in Table 2. A 100-fold purification was achieved with an over-all yield which varied between 10 and 20 per cent.

Properties of the growth factor: The biological activity of the purified material

was nondialyzable, heat labile (5 min at 90°), destroyed by acid (0.1 *N* HCl for 1 hr at 26°), and stable to alkali (0.1 *N* NaOH for 1 hr at 26°).

The ultraviolet absorption spectrum of the material showed a peak at 279 mμ, a trough at 251–252 mμ, and a 280/260 absorption ratio of 1.53.

TABLE 2

PURIFICATION OF THE NERVE GROWTH FACTOR FROM THE SUBMAXILLARY GLAND

Fraction	Total Protein, mg.	Protein Required for 3+ Response, μg per ml
Homogenate	1,800	1.5
Alcohol	450	0.6
Ammonium sulfate	190	0.3
CM-1	146	0.3
DEAE	54	0.15
CM-2	14	0.045
CM-3	3	0.015

The preparation of each fraction is described in the text.

The behavior of the material was examined in a Spinco analytical ultracentrifuge using a Waugh-Yphantis partition cell. The result is shown in Figure 1. Only a single peak was detectable, with an s_{20} of 4.33 S; the molecular weight was estimated to be on the order of 44,000. The centrifugation was continued until the boundary passed into the lower segment of the cell. The concentration of protein in the upper compartment was found to be 4 per cent of that of the starting material, and it contained approximately 4 per cent of the biological activity. The remaining protein and biological activity was recoverable from the lower compartment.

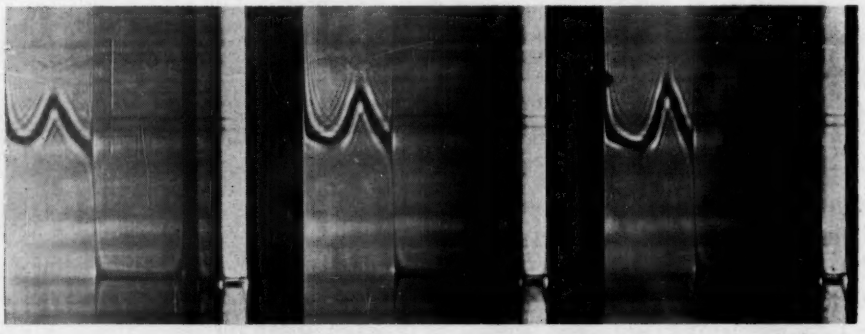

FIG. 1.—Ultracentrifugation pattern of the purified protein at 59,780 rpm. The protein concentration was 2.6 mg per ml. in 0.1 *N* NaCl containing 0.01 *M* phosphate buffer, pH 7.6. The pictures were taken at 8 minute intervals.

Upon acid hydrolysis (6 *N* HCl for 10 hours in an autoclave) and two-dimensional paper chromatography of 150 μg aliquots, the amino acid pattern obtained was found to be qualitatively identical to a similar chromatogram prepared with crystalline bovine albumin. Less than 0.3 per cent hexose was present in the preparation as determined by the orcinol procedure with galactose as a standard at 540 mμ.[14]

Two additional lines of evidence support the view that the salivary gland factor, like the snake venom factor, is a protein. First, the biological activity is destroyed

upon incubation with proteolytic enzymes, and second, the salivary factor is antigenic and the antiserum thus obtained inhibits its biological activity.

The biological activity of the purified material is completely destroyed upon incubation with crystalline pepsin or chymotrypsin. In these experiments 40 μg of the CM-3 fraction were incubated with either 0.4 μg of pepsin in 0.2 ml of 0.02 *M* sodium acetate buffer pH 3.3, or 8 μg of chymotrypsin in 0.2 ml of 0.02 *M* phosphate buffer pH 7.6. The incubations were carried out for 4 hr at 30° and aliquots then were assayed for biological activity. Under these conditions, over 95 per cent of the activity was destroyed, whereas no diminution of activity was detected in control tubes lacking the enzymes. Other controls were run in which additional amounts of the growth factor were added to the enzymatic digestion mixtures after incubation had been completed. No inhibition of the added factor was detected, indicating that the digestion mixture did not interfere with the assay system. Crystalline trypsin was less effective in destroying the biological activity. Under conditions identical to those of the chymotrypsin experiment, 8 μg of trypsin only destroyed approximately 50 per cent of the activity.

The preparation of a specific antiserum and its biological effects are discussed in a later section.

Comparison with the snake venom factor: Although the biological properties of the previously isolated venom protein and the mouse salivary gland protein are very similar, the specific activity of the most purified mouse factor was approximately 10 times greater than that of the best venom preparation. Other differences are also apparent. A comparison of the isolation procedures will show that their behavior on the ion-exchange columns was quite different. The ultracentrifugation pattern of the venom protein showed a single peak with an s_{20} of 2.2 S, whereas the s_{20} of the mouse protein was found to be 4.33. It should be remembered, however, that during the purification of the venom protein, but not of the mouse factor, a 90-min exposure to urea was included. There also appear to be antigenic differences between the two proteins as described in a later section. The biological activities of both proteins were resistant to the rather drastic alkali treatment used.

Biological activity of the growth factor: It has been reported that in tissue culture the salivary gland factor will stimulate the growth of nerve fibers from sensory ganglia isolated from chick, mouse, and rat embryos.[1,2] It will be shown in a separate communication (R. Levi-Montalcini) that sensory ganglia isolated from human embryos will also respond.

Previous tissue culture experiments, using the snake venom protein, have shown that concomitant with the stimulation of nerve fiber outgrowth, there is an increased incorporation of lysine into protein and of adenine into ribonucleic acid. No data were presented to distinguish between net synthesis and turnover.

In the present experiments it is shown that the injection of the salivary gland growth factor into newborn mice results in a marked net increase in the protein, ribonucleic acid, and desoxyribonucleic acid content of the superior cervical ganglion of the mouse. The details of the experiment and the results are shown in Table 3. It may be seen that after 12 days of injection, the protein content of the experiment was more than six times greater than that of the control ganglion, and more than two times greater than that of the normal adult (male). The DNA

content of the experimental ganglion was approximately twice that of the control and again much greater than that of the normal adult. The approximate RNA phosphorus content of the ganglia may be estimated by subtracting the average DNA phosphorus content of the ganglion (fifth column, Table 3) from the total nucleic acid phosphorus (fourth column, Table 3). The RNA value for the experimental ganglia (0.39 μg) is approximately three times greater than that of the control ganglia (0.12 μg) and more than twice the value for the normal adult (0.16 μg).

The detailed cellular changes accompanying these gross analytical differences are described in a separate paper (R. Levi-Montalcini).

The injection of the CM-3 fraction at the levels indicated in Table 3 did not result in any observable difference in the body weight from that of control mice or in other symptoms. However, attempts to inject less purified fractions resulted in a variety of side effects. The daily injection of the ammonium sulfate or CM-1 fractions (Table 2) into newborn mice resulted (in addition to the effect on the nerve cells) in the following gross anatomical changes: (*a*) precocious opening of the eyes (at 6–7 days instead of the usual 12–14 days); (*b*) precocious eruption of the teeth

TABLE 3

EFFECT OF THE INJECTION OF THE GROWTH FACTOR ON THE PROTEIN, RNA, AND DNA CONTENTS OF THE SUPERIOR CERVICAL GANGLION OF THE NEWBORN MOUSE

		Content per Ganglion*		
Age	Treatment	Protein, μg	Total Nucleic Acid, μg P	DNA, μg P
12 days	Control	15 (13–17)	0.18 (0.17–0.20)	0.056 (0.048–0.062)
12 days	Experimental	98 (86–106)	0.51 (0.46–0.57)	0.12 (0.10–0.13)
Adult	Control	36 (33–40)	0.23 (0.21–0.25)	0.066 (0.053–0.076)

Daily subcutaneous injections of 10 μg. of the CM-3 fraction per gm. body weight were made into the experimental newborn mice.

* Each set of values shown is the average obtained from four experimental animals. The figures in parentheses show the range of values obtained. The protein and RNA analyses were carried out on the same ganglion. The DNA analyses were made on separate ganglia of identically treated mice.

(at 5–6 days instead of the normal 8–10 days; (*c*) a marked stunting of the animals with an inhibition of normal hair growth. These results suggested the presence of other physiologically active components in the submaxillary gland of the adult mouse. Preliminary experiments indicate that the factor(s) responsible for these effects is heat stable but is nondialyzable, and is precipitable with ammonium sulfate. It is completely separable from the nerve growth factor.

Preparation and properties of a rabbit antiserum to the growth factor: One mg of the CM-3 fraction was injected into the foot pads of each of two rabbits using Freund's adjuvant (0.5 ml of isotonic sodium chloride containing the antigen, 0.4 ml of Bayol F with 40 μg of Mycobacterium butyricum, and 0.1 ml of Arlacel A). The antibody titer was assayed weekly. The assay, based on the inhibition of the biological activity of the growth factor in tissue culture, was performed as follows: 0.2 μg of the most purified material (CM-3 fraction) in 0.2 ml of isotonic sodium chloride was incubated with 0.2 ml of a series of two-fold dilutions of the rabbit serum. The mixture was incubated at 26° for 1 hr, diluted to 1 ml with isotonic sodium chloride and aliquots were tested for nerve growth promoting activity in tissue culture.

In this manner antibody was detected in both injected rabbits. Rabbit A reached a maximum titer in 4 weeks. The biological activity of 0.2 μg of the growth factor was completely inhibited by 0.001 ml (or more) of antiserum. The maximum titer in rabbit B was reached in 6 weeks; the titer of the antiserum was approximately one-half of that in rabbit A. The rabbits have thus far been kept for 10 weeks following the initial injection with no detectable decrease in titer. Serum obtained from both rabbits prior to immunization showed no inhibitory effects. In all cases the animals were bled from the ear vein.

The following exploratory experiment indicated that the observed inhibition of biological activity was due to the presence in the antiserum of normal precipitating antibodies. To a series of tubes containing 1 ml of antiserum (rabbit B) were added increasing amounts (1–10 mg) of crude antigen (alcohol fraction, Table 2) in isotonic sodium chloride. The mixture was allowed to stand at 26° for two hr and then the precipitate which had formed was centrifuged. Aliquots of the supernatant fluid were assayed for biological activity. Activity was absent in all tubes containing 3 mg or less of the crude antigen. The precipitate (from the 3 mg tube) was washed twice with isotonic sodium chloride and finally dispersed in 0.9 ml of distilled water. Then 0.1 ml of 1 *N* NaOH was added and the solution was allowed to stand for 1 hr at 30°. It was neutralized with 1 *N* HCl to pH 7 using phenol red as an internal indicator. Over 50 per cent of the initial activity was recovered in this solution. Control solutions to which alkali had not been added were inactive. Since a similar alkali treatment of the growth factor did not alter its biological activity or its ability to be inhibited by the antiserum, it appears that the regeneration of biological activity by treatment of the antigen-antibody precipitate with alkali was due to alterations of the antibody molecules. No precipitation occurred in control experiments using normal rabbit serum.

From preliminary experiments, there also appears to be some cross reactivity between the rabbit antiserum and the nerve growth promoting protein present in snake venom. Defining one unit of biological activity as that amount/ml required to show a 3+ response in tissue culture, 1 ml of the antiserum inhibited the biological effect of 83 units of snake venom activity (500 μg of crude venom, Agkistrodon piscivorus). This mixture, although unable to inhibit the biological effect of any further additions of venom, was still capable of neutralizing the effect of approximately 8,000 units of salivary protein (120 μg of the CM-3 fraction).

We have previously reported that the nerve growth promoting effect of snake venom was inhibited by a commercial preparation of antisnake venom serum. 100 μg of the antiserum preparation inhibited 50 μg (approximately 8 biological units) of the crude venom. In the quantities employed, the preparation did not inhibit the biological effect of the mouse salivary factor. In these experiments, amounts of antivenom up to 1,000 μg had no inhibitory effect on 4 biological units of activity derived from the mouse.

Effect of antiserum on the sympathetic chain ganglia: Up to this point in our studies there was no evidence that the salivary growth factor played any role in the normal maintenance of the sympathetic system. If such a factor was present in the general circulation of the normal animal, it was conceivable that its removal or depletion by the injection of the specific antiserum might result in some observable effect on the nerve cells.

The daily subcutaneous injection of the antiserum from rabbit A into newborn (or adult) mice at a level of 0.05 ml per 1.5 gm of body weight resulted in a rapid and extensive destruction of the nerve cells of the sympathetic chain ganglia. The injection of normal rabbit serum had no effect. No external abnormalities were observed in the mice, and after 21 days of injection the body weights of the experimental and control mice (either injected with normal serum or uninjected) were very similar.

The biological evidence for the destruction of the sympathetic system is presented in a separate communication (R. Levi-Montalcini, these PROCEEDINGS, this issue). The same antiserum causes very similar destructive effects when injected into the newborn rat, cat, or rabbit. These results suggest that there is in the general circulation of the species examined a nerve growth factor similar (identical?) in chemical structure and biological activity to that isolated from the mouse submaxillary gland. It is also possible that the antiserum interacts with the antigen (or antigen-like material) on the surface of or within the nerve cell.

The finding that the antibodies produced in the rabbit destroy the rabbit's own sympathetic chain ganglia makes the question of the identity of the mouse salivary factor with the hypothetical rabbit growth factor of great interest.

The role of the salivary glands with respect to the nerve growth factor is not clear. The possibility that the growth factor is produced elsewhere in the organism and is merely concentrated and stored in the salivary glands must be considered (see R. Levi-Montalcini and B. Booker, this issue).

Summary.—A protein with specific nerve-growth stimulating properties has been purified from the submaxillary gland of the mouse. Upon examination in an ultracentrifuge, only one component was detectable with a sedimentation constant of 4.33 S. The protein, when injected into mice, resulted in a marked increase in the protein, RNA, and DNA contents of the sympathetic chain ganglia. A rabbit antiserum to the growth factor was prepared and was found to inhibit the effect of the growth factor in tissue culture and to have specific neurocytotoxic activity when injected into a variety of mammalian species.

The author is greatly indebted to Dr. A. Holtzer for the ultracentrifugal analysis, to Dr. H. Eisen for aid in the preparation of the antiserum, and to Dr. E. Robins for aid in the DNA analyses. The technical assistance of Miss Jean Macnish is gratefully acknowledged.

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*THE ACCOMMODATION OF NONCOMPLEMENTARY BASES IN HELICAL POLYRIBONUCLEOTIDES AND DEOXYRIBONUCLEIC ACIDS**†

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It is now well established that deoxyribonucleic acid* is the molecular counterpart of the genetic apparatus. In this two-stranded helical polynucleotide the perfect crystalline geometry which normally prevails requires that adenine residues in one chain be hydrogen-bonded to thymine residues in the other; similarly, guanine residues can only be hydrogen-bonded to cytosine residues.[1–3] Thus, one chain is necessarily the complement of the other, and it appears likely that each chain serves as the template for the synthesis of its complement.[4–6]

Current views on the molecular basis of mutations hold that they are due to a variety of alterations in the nucleotide sequence within the two-stranded DNA helix. Presumably, many of these alterations arise from mistakes made during its replication. It appears from studies of fine structure genetics[7,8] that such mistakes could involve the substitution of noncomplementary bases, and also the addition or deletion of one or a sequence of nucleotides, during the synthesis of a progeny chain on its template. In view of these plausible mechanisms of mutation, we have examined the possibilities whereby noncomplementary base pairs can be accommodated within the two-stranded helical framework of DNA. We first investigated the effects of varying proportions of noncomplementary bases incorporated into pairs of complementary polyribonucleotides which are known to form two-stranded helices like DNA. The results of such experiments allowed us to exclude the notion that noncomplementary bases remain within the interior of the helix. Instead, they suggested that noncomplementary nucleotides of opposite chains rotate out of the helix in such a manner as to enable normal hydrogen-bonded base pairing of subsequent regions of nucleotides which are in register. This structural alternative was then explored by model building and found to be readily plausible.

Experimental Approach.—Polyadenylic and polyuridylic acids* are single stranded polyribonucleotides[9, 10] containing base residues equivalent to the complementary pair, adenine-thymine, present in DNA. Indeed, in neutral saline solution they form a 1 : 1 two-stranded helical complex which has essentially the same structure as DNA.[11–13] Under appropriate conditions, a second strand of poly U can be induced to wrap around this poly(A+U) helix, so that a three-stranded complex is formed in which the molar ratio of adenine to uracil is 1 : 2.[13]

Mixing curves: The interaction of polynucleotide strands to form helical complexes is accompanied by a marked decrease in ultraviolet absorbance.[10, 11, 13, 15] For long chains, this hypochromic change can be expected to reflect in a linear manner the number of nucleotides involved in the interaction. Thus, by titrating one species against another, it is possible to locate the mixture of maximum hypochromicity, and thereby to accurately determine the molar ratio of the reacting species in the complex being formed.[13]

In the present investigation, such mixing curves were used in order to study quantitative aspects of the interaction of poly A or poly U with a series of single stranded poly AU copolymers.[16] Complex formation here necessitates juxtaposition of the mismatched bases, adenine-adenine and uracil-uracil, at certain levels of the helix. The manner in which these pairs are structurally accommodated will influence the molar ratio at the point of maximum hypochromicity.

The underlying assumption behind these interaction experiments is, of course, that polynucleotide chains have the capacity to explore and find the partners which permit the complete fulfillment of their hydrogen bonding potentialities. This was previously shown for the interaction of poly A and poly U.[13, 17] The results to be described below also bear out this assumption. Even those single-stranded AU copolymers which exhibited a high degree of intramolecular hydrogen bonding (as evidenced by a large reversible hyperchromic change on heating) were found to interact optimally with homopolymer to form multistranded complexes.

Absorbance-temperature profiles: Helical macromolecules, being highly ordered, are found to denature or "melt" over relatively narrow temperature ranges.[18, 19] In the case of polynucleotides, the profile of this transition can be followed spectrophotometrically by measuring the increase in ultraviolet absorption (reversal of hypochromicity) as a function of temperature. In a given solvent, the temperature of the midpoint of this transition (T_m) will reflect the relative stability of the complex.[15] This property was assessed for the complexes formed between poly A or poly U and several poly AU copolymers in order to evaluate the effect of the mismatchings on the strength of the interaction.

Methods.—Polynucleotide samples: Samples of poly A and poly U, and samples of poly AU differing in their content of adenylic and uridylic residues were synthesized in this or other laboratories as previously described,[20] employing the reaction catalyzed by polynucleotide phosphorylase.[21]

Polymers were generally isolated from their polymerization reaction mixtures by precipitation with ethanol, and were purified by reprecipitation with ethanol from clarified neutral saline solution, followed by extensive dialysis against neutral saline containing versene or citrate. Stock solutions of polymers were stored at −20°C.

Base analysis of polymers: Duplicate determinations of the ratio of adenylic to uridylic residues in the copolymers were made according to a modification of a method of Markham and Smith.[22] About 0.5 mg of polynucleotide was hydrolyzed in an alkali resistant glass test tube in 0.4 *N* NaOH at 37°C for 18 hours. The hydrolysate was neutralized and divided into two parts for chromatography on Whatman No. 1 paper. Descending chromatograms were developed at 4°C with a solvent containing saturated aqueous $(NH_4)_2SO_4$ [79 vol], water [19 vol.], and isopropanol [2 vol.]. At this low temperature sharper spots were obtained, so

long as the chromatography paper was dry when the run was started. After thorough drying of the paper, the adenylate and uridylate spots and corresponding paper blanks were eluted quantitatively and their amounts determined spectrophotometrically.

Polymer concentration determination: Duplicate aliquots of stock solutions used for mixing curves were hydrolyzed in 0.4 *N* NaOH at 37°C for 18 hours. It was found necessary to incubate similar flasks containing 0.4 *N* NaOH in order to provide proper blanks. The hydrolysates were then diluted to a constant volume and final acid concentration of 0.1 *N* with HCl. Knowing the nucleotide composition and the extinction coefficients of the monomers at 259 mμ, the molar nucleotide residue concentration of the homopolymers and copolymers was calculated from the absorbance at 259 mμ.

Mixing curves: Polymer stock solutions were made up to the same nucleotide residue concentration. For two-stranded complex formation, the solvent used was either 0.15 *M* NaCl + 0.015 *M* sodium citrate, pH 7, or 0.15 *M* NaCl + 0.045 *M* sodium cacodylate, pH 6.9—the latter being preferred because of its ability to deter bacterial contamination. For each homopolymer-copolymer combination, a series of mixtures was made such that the total number of nucleotide residues was kept constant while the ratio of homopolymer to copolymer was varied. These mixtures were allowed to equilibrate at a constant temperature, and their absorbance at 259 mμ then determined, against a solvent blank, in a Beckman DU spectrophotometer adapted with a thermostated cell chamber. Additional mixtures, with polymer ratios clustered about the observed minimum, were then used to precisely locate the optimally interacting mixture. To detect any hypothetical three-stranded structure which might form, similar mixing curves were performed in solvent also containing 0.05 *M* $MgCl_2$.

TABLE 1

ANALYTICAL PROPERTIES OF SINGLE-STRANDED POLYRIBONUCLEOTIDES

Polymer	Mole % Adenylic Residues	$S^\circ_{20,w}$*	Approximate Molecular Weight
Poly A	100	9.0	600,000†
Poly U	0	4.3	150,000†
Poly AU "90"	90	3.0	50,000†
Poly AU "66"	66	1.4	8,000‡
Poly AU "53"	53	3.9	40,000‡
Poly AU "37"	37	2.9	25,000‡

* These mean sedimentation constants were determined in 0.15 *M* NaCl + 0.015 *M* sodium citrate, pH 7.
† Extrapolated from S° versus M.W. calibration curve for Poly A.
‡ Extrapolated from S° versus M.W. calibration curve for RNA.

Absorbance-temperature profiles: Absorbance measurements were made at 259 mμ on solutions contained in quartz stoppered cuvettes with a thermostated Beckman DU spectrophotometer controlled to ±0.3°C.

Sedimentation constants: Sedimentation velocity measurements were made at about 22°C in a Spinco Model E Ultracentrifuge using the ultraviolet absorption optical system. The mean $S^\circ_{20,w}$ was calculated from the moving boundaries traced with a Spinco analytrol.

Model building: Pauling–Corey space-filling nucleotide models,[23] (1″ = 2 Å) were used to explore possible configurations for the helical homopolymer-copolymer complexes.

Results.—Properties of polymers: In Table 1 are presented some analytical properties of the single-stranded polynucleotides used in the interaction studies. The compositional data are subject to an average deviation of ±1.5 per cent. Note that the identification number following each copolymer is derived from the mole per cent of adenylic residues that it contains. Approximate molecular weight values were derived from the sedimentation constants using either the calibration scale of S° versus molecular weight for RNA[24] or that for poly A.[9] It is recognized that these extrapolations may be subject to errors of as much as 50 per cent. Nevertheless, it is apparent that all the samples are of sufficiently high molecular weight so that hypochromicity can serve as a linearly proportional measure of complex formation.

Homopolymer-copolymer interactions: A priori, one might conceive of two possible ways in which noncomplementary base pairs could be accommodated into the DNA type of helical structure.

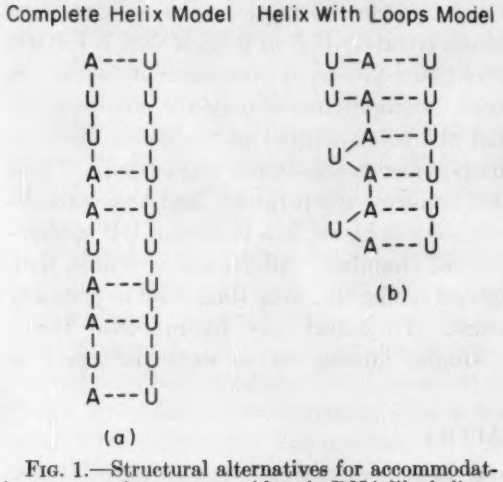

FIG. 1.—Structural alternatives for accommodating noncomplementary residues in DNA-like helices. (*a*) In any case of the Complete Helix Model the molar ratio of poly U:poly AU will always be 1:1 for the two-stranded complex. If a second strand of poly U is added, this ratio becomes 2:1. (*b*) In the Helix-With-Loops Model the molar ratio of poly U:poly AU varies with copolymer composition. In the example shown, this ratio is 6:10 for the two-stranded complex and 12:10 when a second strand of poly U is added.

The first possibility is that such nonmatching bases could be held *within* the helix either with or without hydrogen bonding. Conceivably this would involve occasional distortions of the helix to compensate for the steric variations involved in pyrimidine-pyrimidine and purine-purine pairings.

The alternative is that these noncomplementary nucleotide residues could in some manner rotate out of the helix to allow a continuity of complementary base pairs along its entire length.

These two possibilities are illustrated schematically for poly (AU + U) in Figure 1*a* and *b*. From Figure 1 it is clear that the overall composition of the two complexes will be different depending on the method by which nonmatching bases are accommodated. If these are retained within the helix (Fig. 1*a* "Complete Helix" model), the mixture of maximum hypochromicity in a mixing curve will always contain equimolar amounts of homopolymer and copolymer for the two-stranded complexes poly (AU + A) and poly (AU + U). In the alternative case, where the nonmatching residues in the copolymer do not reside within the helix (Fig. 1*b* "helix-with-loops" model), the homopolymer content of the complex will be *less* than 50 moles per cent. As is apparent from Figure 1 (*b*), the exact composition of such a complex can be calculated from the proportion of noncomplementary bases to be accommodated. If complex formation is complete, this scheme predicts that the mixing curve minimum will lie at the point where the

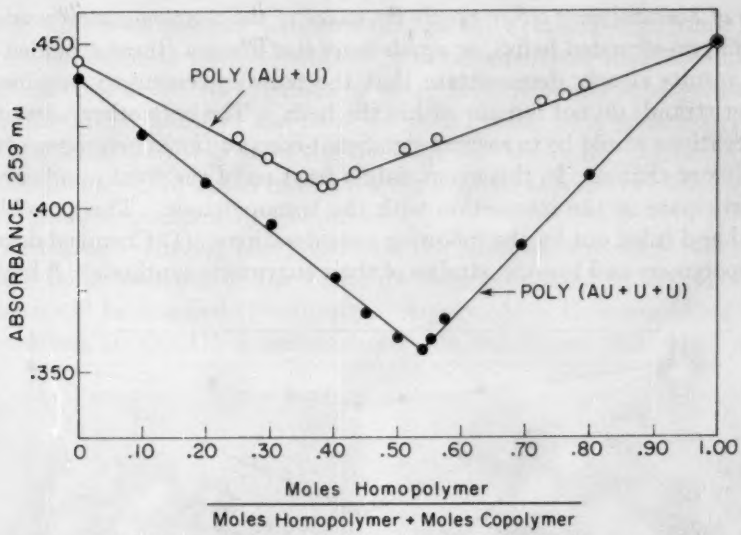

FIG. 2.—Mixing curves for poly AU "66" + poly U. *Open circle* points are for two-stranded complex formation at 21°C in 0.15 *M* NaCl + 0.045 *M* cacodylate, pH 6.9. *Filled circle* points are for three-stranded complex formation at 17°C in the same solvent + 0.05 *M* $MgCl_2$. The composition at the point of maximum hypochromicity did not change when the temperature was lowered to 4°C.

moles of homopolymer equals the moles of *complementary* residues in the copolymer.

Similar considerations can be used to analyze mixing curves involving the formation of the three-stranded complexes, poly (AU + U + U).

Mixing curves, such as those in Figure 2, were obtained on combinations of the copolymers and poly U or poly A. The composition of the various homopolymer-copolymer complexes were determined from the mixtures of maximum hypochromicity, and are compared in Table 2 with the theoretical values calculated for the two models described above. Two sets of the data included were derived from mixing curves published in another connection by Steiner.[25] It can be seen from Table 2 that in every case where two- or three-stranded complexes were found, their compositions agreed very closely with those expected for the model of the helix-with-loops. This is also evident from the examples in Figure 3 which show that *in every combination, maximum complex formation occurred at the point where*

TABLE 2

COMPOSITION OF HELICAL POLYRIBONUCLEOTIDE COMPLEXES

	Mole % of Homopolymer in Mixture of Maximum Hypochromicity					
	2-Stranded Complexes			3-Stranded Complexes		
		Theoretical			Theoretical	
Polymer Mixture	Experimentally Found	Helix With Loops	Complete Helix	Experimentally Found	Helix With Loops	Complete Helix
Poly A + Poly U	50	X	50	67	X	67
Poly AU "90" + Poly U	...	..	..	65 ± 1	64.5	67
Poly AU "66" + Poly U	38 ± 1	40	50	54 ± 1	57	67
Poly AU "53" + Poly U	34 ± 1	34.5	50	...	..	..
*Poly AU "62" + Poly U	...	..	..	57	55	67
*Poly AU "82" + Poly U	...	..	..	64	62	67
Poly AU "37" + Poly A	38 ± 3	39	50	X	X	X

* These sets of data were derived from the work of Steiner.[25]
Dots in place of a value indicate the data were not available, while an *X* indicates the value is not meaningful.

the moles of homopolymer either equals the moles of the complementary residue in the copolymer (two-stranded helix), *or equals twice that amount* (three-stranded helix).

These results clearly demonstrate that the noncomplementary residues of the copolymer strands do not remain within the helix. The only other way to explain our observations would be to assume significant compositional heterogeneity among the copolymer chains. In this event only a fraction of the total number of chains could participate in the interaction with the homopolymer. This possibility was evaluated and ruled out by the following considerations: (1) Chemical degradation of AU copolymers and isotopic studies of their enzymatic synthesis[20, 26] have shown

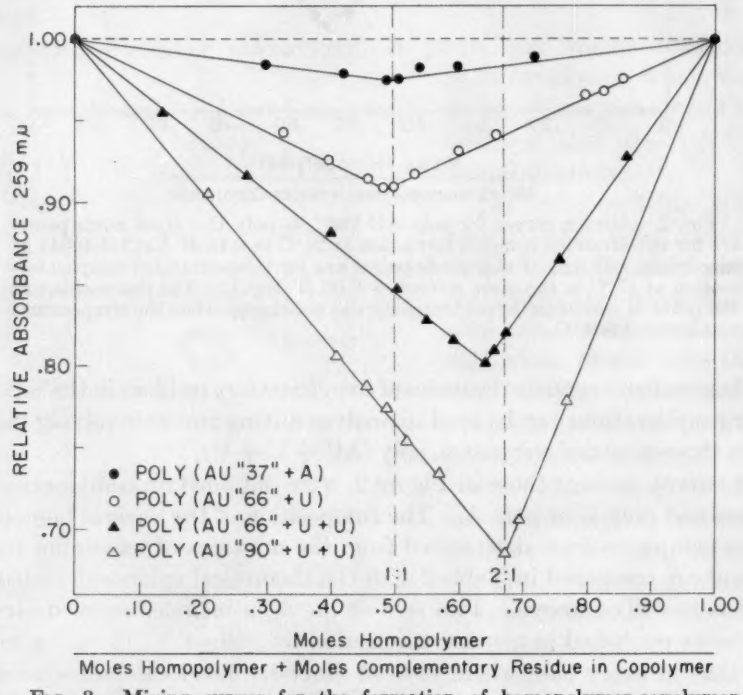

FIG. 3.—Mixing curves for the formation of homopolymer-copolymer polyribonucleotide complexes. Relative absorbance is the ratio of the absorbance of the mixture to that of the average of the unmixed constituents. It should be noted that the depth of these mixing curves will depend in part on the initial hypochromicity of the reacting species. The extreme shallowness of the (AU "37" + A) curve can be explained by the fact that both poly A and the AU copolymers are highly hypochromic, whereas poly U is not.

that the sequence of adenylic and uridylic residues is essentially random. Moreover, the chain lengths of our samples are sufficient to average out small compositional differences. (2) The self-consistency of all the interaction data argues strongly for a quantitative reaction. It is especially reflected by the equivalence of the amount of homopolymer required to form the poly (AU "66" + U) two-stranded complex, and the additional amount required to form the three-stranded complex. This rules out the possibility that the original copolymer solutions contained any stable two-stranded helical complexes which could not be titrated. (3) The fact that the hypochromicity increases in a *linear* manner as the copolymer

is titrated with homopolymer indicates that all the available copolymer molecules have an equal capacity for interaction. If some copolymer molecules had a preferential tendency to interact because of more favorable residue sequence or composition, the initial phase of the titration would result in a greater hypochromic change than that occurring when the titration was nearer to completion. In this event, the mixing curve would have a readily detectable concave character extending to the point of maximum hypochromicity.

Model building: Having concluded that the noncomplementary residues in the copolymer must loop out of the helix, we then proceeded to examine how this condition could be satisfied structurally. A poly (A + U) space-filling helix was built according to the DNA coördinates.[27] It was found that when a uridylic

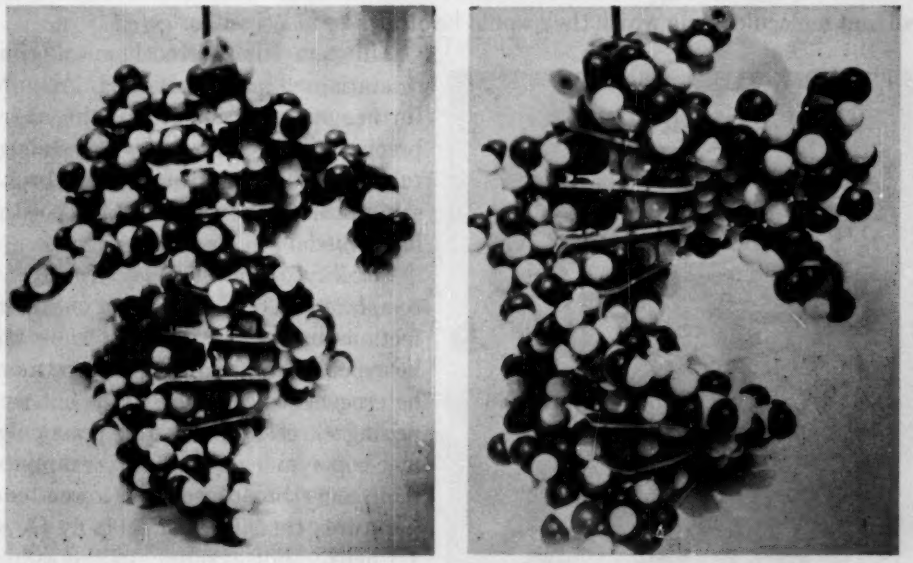

FIG. 4.—(*a*) A poly (A + U) helix containing a single nucleotide loop in each chain. This portrays the situation which might occur in DNA if cytosine and thymine are bases located in opposite chains at the same level, while all the other residues are in register. Note that the loops point away from the backbone and do not obstruct either groove.

FIG. 4.—(*b*) A poly (A + U) helix containing in the A-chain a looped region of two uridylic residues.

residue is incorporated into the A-chain, it can be readily looped out of its position within the helix, merely by rotation about its two adjacent phosphodiester bonds. This leaves the uracil base pointing radially out from the helix axis, and allows an adjacent nucleotide residue to move into the vacancy created without strain. (Fig. 4*a*). In addition, similar loops containing two, three or more nucleotides are also possible (Fig. 4*b*, *c*). Indeed, there appears to be no structural limitation on the number of nucleotide residues which can be incorporated into a loop. It should be noted, however, that whereas a loop of one residue is rigidly fixed in position except for the rotation about its phosphodiester bonds, larger loops become increasingly more flexible.

Exactly the same considerations are involved for any other noncomplementary base residues which might be incorporated into either chain. In all cases, the

regularity of the helix in the vicinity of the loop is unaffected, both the 3.4 Å separation and 36° translation of successive nucleotides being maintained. Furthermore, the loops do not interfere with either the "deep" or "shallow" groove, which explains why it is possible to add a second poly U chain to the poly (AU + U) complexes.

The only significant alteration in the helical backbone is that for each loop the separation of one pair of PO^- groups is reduced from 7 Å to approximately 6. However, it is apparent that other thermodynamic considerations such as the maximization of hydrogen bonding and the increased entropy brought about by loop formation tend to offset this small increase of charge repulsion. An additional factor favoring loop formation would seem to be that removing noncomplementary bases from the interior of the helix enables them to form hydrogen bonds with solvent molecules from which they would be otherwise inaccessible.

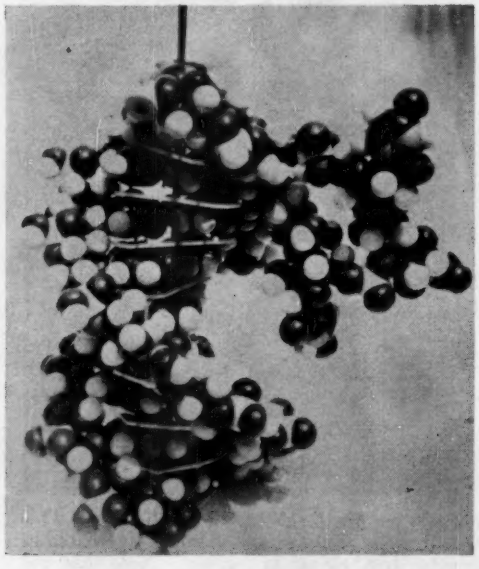

FIG. 4.—(*c*) A poly (A + U) helix containing in the A-chain a looped region of three uridylic residues.

Although the stereochemical considerations just described require further examination with the more precise models in use by crystallographers, it would appear that the broad structural features of the helix-with-loops model are correct.

Stability of homopolymer-copolymer complexes: The introduction of imperfections into a two-stranded helix can be expected to produce a weaker, more heterogeneous complex. It is not surprising therefore that the homopolymer-copolymer complexes examined melt more broadly and at lower temperatures than the perfect poly (A + U) helix. For example, in the saline-citrate solvent, whereas poly (A + U) melts almost entirely between 57 and 62°, poly (AU "53" + U) melts from 5 to 46° and has a T_m of about 24°.[28] The latter helix contains 31 per cent unpaired, looped out bases. Since its complete formation requires several hours at 2°, it would seem that a limit to the number of tolerable imperfections is being approached under these experimental conditions. In fact, for an AU copolymer containing 51 per cent adenylic residues, no interaction with poly U could be observed. In any case, the number of mismatchings which can be incorporated into a DNA-like helix is surprisingly large.

Discussion.—The helix-with-loops model adds a dimension of freedom to configurational considerations of helical polynucleotides. In the case of RNA, the model offers a means for satisfying the requirement for the short helical regions which have been recently suggested.[15] In addition, it involves the generation of a new type of tertiary structure which could have biological significance. A molecular structure for RNA incorporating these features will be described in a subsequent report.

There are several contexts in which the helix-with-loops model might be significant for DNA. In view of the similarity of essential structural features between the poly (A + U) helix and DNA, it seems likely that any base pair mismatchings arising during DNA synthesis would also be accommodated by loop formation. From the foregoing it is apparent that the looping mechanism does not require any significant alteration in the structural parameters of the poly (A + U) helix. It also does not result in significant crowding of the grooves, which in the case of DNA must remain unobstructed in order to contain protamine or histone.[29] The homopolymer-copolymer system does not provide an entirely analogous model to a DNA molecule with defective base pairs. Since one of the strands of the synthetic helix is a homopolymer, this strand need never form a loop in order to keep all nucleotides in register. Further, this helical complex is the resultant of an interaction between two *already existing* polynucleotide chains. In DNA, on the other hand, not only is there a specific sequence of residues, but it is believed that the helix is formed by one chain being synthesized in a linear fashion upon its complementary template chain.[4-6] Under these conditions, the accidental addition to the growing chain of a nucleotide which is not complementary to its partner in the template chain will lead to one of three obvious consequences. Any of these would appear to be a possible molecular counterpart for point mutations. These models showing how mutational events might be accommodated are represented schematically in Figure 5.

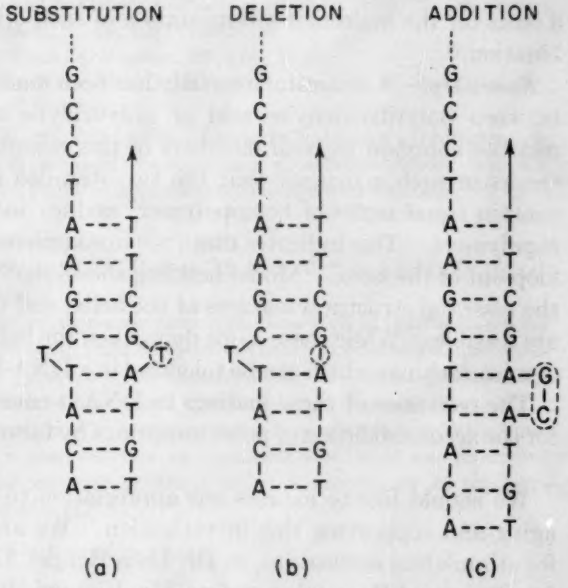

FIG. 5.—Hypothetical models for point mutations. In each case, the template chain is the one on the left. Note that while the templates are identical in each case, the sequence in the growing chains varies, depending on the kind of "mistake" made during replication. The "mistake" in the growing chain is indicated by a dotted circle.

In the first case, Figure 5*a* (compare with Fig. 4*a*), both the erroneous base and its noncomplementary partner have looped out of the growing helix, thus enabling the succeeding regions to pair normally. This type of accommodation merely involves the *substitution* of one residue for another, and results in no difference in chain length between the chains.

In Figure 5*b*, however, the erroneous base has paired with a succeeding base on the template, causing the intervening template residue(*s*) to loop out of the helix. Chain growth then continues, leading to a progeny chain which is shortened by one or more residues. Conceivably, such a *deletion* in the growing chain could also be caused by the presence of a pre-existing distortion in the template chain, rendering some of its residues inaccessible.

In the last case, Figure 5*c*, the errors made on the growing chain have been removed from the helix to enable succeeding chain growth to continue normally. This *addition* of residues to the growing chain results in a progeny chain longer than its template.

While it would seem that the *substitution* mechanism could produce reversible mutations (including those induced by base analogues in bacteriophage[7, 8]), the *addition* and *deletion* mechanisms could account for those mutational events which have been found to be irreversible.

It is worth noting that these models for point mutations can serve equally well to explain the incorporation into DNA of unnatural or unusual nucleotides incapable of forming satisfactory hydrogen bonds with any of the naturally occurring residues.

Finally, it is important to recognize that the helix-with-loops model may provide a basis for the molecular interpretation of such other genetic phenomena as recombination.[30]

Summary.—A quantitative study has been made of the helical complex formation between polyriboadenylic acid or polyuridylic acid and copolymers containing residues common to both members of this complementary pair. The interaction occurs in such a manner that the two-stranded helical complexes formed always contain equal moles of homopolymer residues and complementary residues in the copolymers. This indicates that the noncomplementary residues in the copolymers loop out of the helix. Model building shows that this can be done without altering the essential structural features of the helix, and that loops of one or more residues are feasible. While these loops do weaken the helix, it is apparent that the number of mismatchings which can be tolerated in a DNA-like helix is quite large.

The relevance of these findings to DNA is considered, and hypothetical schemes for the accommodation of point mutations by forming such loops are presented.

We should like to express our appreciation to Professor Paul Doty for encouraging and supporting this investigation. We are also indebted to Dr. E. Freese for stimulating discussions, to Dr. Leon Heppel, Dr. R. C. Warner, and Dr. A. Rich for the poly AU samples, and to Mr. Richard Blake for capable assistance. This work has been aided by grants from The American Heart Association, the United States Public Health Service, C-2170 and the National Science Foundation, G-7849.

* The following abbreviations have been used: RNA = ribonucleic acid; DNA = deoxyribonucleic acid; A = adenine; U = uracil; C = cytosine; G = guanine. The prefix *poly* is used to indicate a homo or copolymer of nucleotide(s) of the indicated base(s). Polynucleotide complexes are indicated by the prefix *poly* followed in parentheses by the summated homo or copolymer chain symbols.

† This paper is No. III in a series entitled *Polynucleotides*, of which the last was by J. R. Fresco, *J. Mol. Biol.*, **1**, 103 (1959).

‡ Established Investigator of the American Heart Association.

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where x = mole fraction of complex melted; t = temperature; a(t) = absorbance of the complex as a function of temperature; b(t) = absorbance of an equimolar amount of non-interacted constituent chains (calculated from their separate profiles); and c = absorbance of the completely formed complex [minimum value of a(t)];

$$a(t) = c[1 - x] + x[b(t)]$$

$$x = \frac{a(t) - c}{b(t) - c}$$

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STUDIES ON SOLUBLE RIBONUCLEIC ACID (S-RNA) OF RABBIT LIVER, I. AMINO ACID ACCEPTOR SPECIFICITY AND MOLECULAR WEIGHT

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Cytoplasmic or soluble RNA (S-RNA) exhibits the unique biological property of functioning as an acceptor for acyl-activated amino acids and as a donor of the amino acyl group to microsomal proteins.[1, 2] It has been established that the amino acid is linked to the polynucleotide chain through the 2′ or 3′ hydroxyl group of the terminal nucleotide having an unesterified 3′ hydroxyl group,[3, 4] and furthermore that the terminal nucleotide is adenylic acid.[3, 5] For a given S-RNA preparation there are a finite number of acceptor sites, presumably one for each polynucleotide chain and it has been shown that various amino acids can be bound independently of one another, but in fixed amounts, to a given S-RNA preparation.[2, 7, 8] From this observation it has been concluded that the number of acceptor sites for any one amino acid is smaller than the total number of acceptor sites. In a few instances it has been possible to achieve a partial separation of the accepting species

TABLE 1

Sedimentation Coefficients Calculated from Partition Cell Experiments*

	$S_{20,w}$	
	C^{14} = RNA − Amino Acid†	O.D. = Total RNA
Leucine	4.4 ± 0.6 (6)	4.3 ± 0.5 (6)
Proline	4.2 ± 0.7 (7)	4.3 ± 0.5 (8)
Valine	4.0 ± 0.7 (4)	4.5 ± 0.7 (4)

* The figures in parentheses indicate the number of experiments which were averaged. All sedimentation coefficients have been corrected to standard conditions. The ionic strength varied from 0.03 to 0.20, the temperature from 2 to 22°C and the concentration of RNA from 0.01 to 0.1 gm/100 ml. None of these variations were found to cause large changes in the measured sedimentation coefficients. The pH was approximately 7.0. No experiments were discarded in calculating the average values given and mean deviations are indicated.

Amino acid-labeled S-RNA was prepared by incubating 15–30 mg of rabbit liver S-RNA,[12] in approximately 10 ml, with the appropriate amino acid activating enzyme[13] in the presence of ATP, 10 mM; $MgCl_2$, 20 mM; NaCl 2.8 mM; K phosphate buffer pH 7.6, 2.8 mM; and C^{14} proline, leucine, or valine, 0.05 mM. The nonreacted amino acid was removed by precipitating the S-RNA-amino acid with 6 per cent perchloric acid and exhaustive acid washing followed by extraction with NaCl-phosphate buffer pH 7.4 and dialysis of the labeled RNA. The S-RNA-amino acid preparation thus obtained is capable, underap propriate experimental conditions, of transferring the bound amino acid to microsomal protein without dilution with free amino acid.

S-RNA from one another.[9, 10] Any given S-RNA-amino acid preparation therefore consists of a mixture of polynucleotide chains only some of which are charged with the specific amino acid while others, which are uncharged, will act as acceptors for other amino acids in the presence of the appropriate enzymes and substrates. Thus, it is established that S-RNA exhibits specificity toward different activated amino acids but the basis for this specificity is unknown.

The possibility that specificity for different amino acids might be related to the length of the polynucleotide chain was investigated in the ultracentrifuge by means of the moving partition cell of Yphantis and Waugh.[11] This technique provides a powerful tool for the study of the relationship between molecular size and biological activity. Inasmuch as each S-RNA species can be charged with an isotopically

labeled amino acid, both the sedimentation coefficient of the amino acid-labeled S-RNA component and that of the total S-RNA present can be determined independently in the same experiment.

The sedimentation coefficients, (in Table 1) were obtained from partition cell experiments using the equation developed by Yphantis and Waugh.[11] The weight average s values for the total RNA present in the solution were calculated from ultraviolet absorption measurements, while the values of s for the individual species were calculated from C^{14} determinations of the same ultracentrifugal samples.

Average values of the sedimentation coefficients of three S-RNA preparations charged respectively with leucine, proline, or valine are listed in Table 1. It is clear that in each case the sedimentation coefficient of the species which carries the amino acid is the same as that of the total S-RNA; moreover, it can be seen that there are no significant differences in s among the three species examined.

On the assumption that the sedimentation coefficient of S-RNA is a measure of its size, it may be concluded from these studies that the specificity of S-RNA as an acceptor for different amino acids is not related to molecular weight. Ultracentrifugal studies with U. V. optics[14] have shown that this preparation of S-RNA exhibits a reasonably smooth distribution of sedimentation coefficients around a single sharp peak at 4.2S, with 85 per cent of material sedimenting with s of less than 8.0. While this distribution of sedimentation coefficients is due in large part to heterogeneity with respect to chain length, the preparation is relatively more homogeneous in this respect than other RNAs.

It would appear therefore that the basis for the biological specificity of S-RNA must reside in its nucleotide composition or in the sequence in which the units are arranged.

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*THE MECHANISM OF BACTERIAL FRAGILITY PRODUCED BY 5-FLUOROURACIL: THE ACCUMULATION OF CELL WALL PRECURSORS**

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Communicated by David Rittenberg, January 22, 1960

In a previous communication we have reported that there is an early phase in the bacterial action of 5-fluorouracil (FU) on *E. coli* K_{12} which produces an "osmotic imbalance" in the cells.[1]

Further study of this phenomenon revealed that FU exerts its lethal action only on growing bacteria.[2] Inhibition of growth during the time of FU treatment by metabolic inhibitors or by omission of growth factors from the media of auxotrophs prevented the development of osmotic imbalance.

When exponentially growing bacteria were treated with FU and incubated on nutrient agar plates for various times, 90–95 per cent of the cells did not give rise to colonies. However, it was possible to recover these cells by a transfer from the nutrient agar plates to sucrose-supplemented agar provided the replica plating was done *before* a critical 25 minutes of incubation on the nutrient agar. After 25 minutes of incubation, 90 per cent of the cells could no longer be recovered by this method, possibly since they had already disintegrated on the nutrient plates.

When the FU-treated bacteria were plated on a thin layer of agar, observation under a phase contrast microscope revealed a series of anomalous cytological changes which culminated in the sudden disintegration of the cells.[3]

The disintegration of FU-treated bacteria could also be demonstrated in fluid medium: after 25–30 minutes of incubation in broth, about 90 per cent of the cells abruptly disintegrated and sedimented out of the medium. The timing of these events suggests that a great part of the population must have been synchronized, possibly by the FU treatment itself (thymine starvation). The disintegration of the cells at the end of a normal generation time suggests a possible connection between the division process and the events of disintegration.

The fate of the bacteria treated with FU depends fully upon the conditions of growth during a critical period *following* the exposure to the drug. Maximum rate of growth during this time inevitably leads to cellular disintegration unless the osmotic pressure of the medium is raised before the cells reach some irreversible stage in the process of disintegration. Suppression of the metabolic rate during this critical period also "rescues" the cells from FU-induced death. This can be accomplished by keeping the nutrient plates at low temperatures for prolonged periods or by plating on minimal medium. Slow rate of metabolism apparently enables the cells to "heal" the lesion introduced by FU and thus to restore full viability. The length of the critical period during which the cells need the protection of high osmotic environment was found to be 60 minutes. There was no cell division during this time.

The prevention of cellular disintegration by high osmotic milieu suggested the involvement of some cell wall abnormality. Our recent findings that FU-treated

cells also exhibit an increased sensitivity toward heat and mechanical stress could also be interpreted as consequences of diminished rigidity of the cell wall. (FU-treated cells also show high UV sensitivity.[4])

In order to test this hypothesis we have searched for the accumulation of cell wall precursors,[5] and found a significant increase of N-acetylhexosamine esters[6] in the FU-treated cells. Table 1 summarizes the results of experiments designed to establish a possible correlation between the accumulation of N-acetylhexosamine esters and the presence of osmotic imbalance in bacteria. The data clearly establish such a correlation.

TABLE 1

Incubation medium*	Supplement added	μM-s of N-acetylhexosamine esters accumulated per 10^{12} cells	Osmotic effect‡
Complete	None	3.8	1
Complete	FU(100 μg/ml)	33.6	21
Complete	FU (5 μg/ml)†	15.0	1.5–2
No glucose	FU(100 μg/ml)	1.0	1
No glucose	None	5.3	1
Complete	FU(100 μg/ml+ uracil (100 μg/ml)	2.0	1
Complete	FU(100 μg/ml+ thymine (100 μg/ml)	28.0	18
Complete	2-thioracil (100 μg/ml)	2.5	1
E. coli, B, complete	FU(100 μg/ml)	1	1

* Bacteria at half-maximum growth in exponential growth phase in synthetic medium (Gray and Tatum[7]) were incubated with FU for 60 minutes at 37°C.

† 5 μg/ml FU is just above the threshold concentration which induces "osmotic imbalance" under the standard conditions of incubation.

‡ Osmotic effect was quantitatively expressed as the ratio of numbers of colonies recovered on sucrose supplemented plates to the number of colonies recovered on nutrient agar plates: S/N; in normal bacteria S/N = 1.

The accumulation showed linear increase with incubation time: 4μM-s at 0 minutes, 8.5 μM-s at 30 minutes and 16 μM-s at 60 minutes of incubation (expressed per 10^{12} cell.). Preliminary experiments with an amino acid auxotroph of *E. coli* K_{12} (C-600-25, Leu$^-$, Threo$^-$) showed that resting populations of these bacteria (produced by amino acid starvation) are also able to accumulate these compounds in the presence of glucose and FU.

In attempts to isolate the accumulated compounds, large batches of FU-treated bacteria were extracted and fractionated on Dowex-1-Cl columns according to the method of Strominger.[6] 90 per cent of the N-acetylhexosamine esters adsorbed were recovered in two peaks, a major and a smaller one, both eluted with 0.05 *M* NaCl–0.01 *M* HCl (Fig. 1). The peaks were located on the basis of UV absorbance and the quantitative Elson-Morgan reaction. No N-acetylhexosamine containing component came off the column with any of the successive eluents.

The two peaks were not homogeneous. Paper chromatography resolved each peak to at least 5 components all having typical nucleotide spectra in the UV. Two of the components of the major peak on strong acid hydrolysis yielded diaminopimelic acid, glutamic acid, alanine, and UV-absorbing spots but no hexosamine. Another component of the same peak on strong acid hydrolysis yielded a UV-absorbing component which had UV spectra and chromatographic mobility identical with those of an authentic sample of FU. The fourth—and largest—component of the major peak contained N-acetylhexosamine, uracil but no amino acids.

It will be of interest to determine at what site FU interferes with the assembly of the cell wall mucocomplex. It appears likely that small quantities of FU become

metabolized to some cell wall precursor and this product may inhibit the whole enzyme complex of cell wall synthesis. This may lead to the accumulation of relatively large amounts of the normal precursor(s). The restoration of cells to viability would then involve the slow removal, by some means, of this interfering compound from the synthetic pathway.

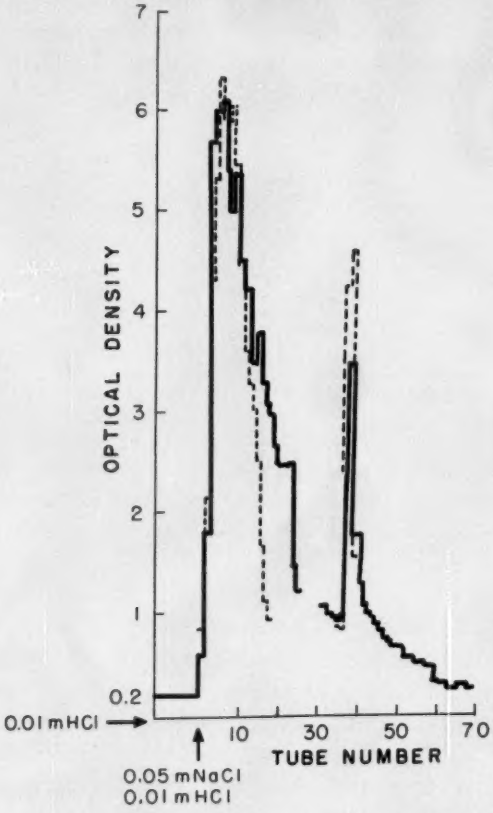

FIG. 1.—Isolation of the nucleotide linked N-acetylhexosamine esters on Dowex-1-Cl. 15 liters of bacteria at half maximum growth in synthetic medium were treated with FU(100 μg/ml) for one hour and then harvested, extracted, and fractionated on an ion-exchange column.[7] The figure only shows the part of elution diagram where the N-acetylhexosamine esters were eluted. Solid lines indicate UV absorbance at 260 mμ, dotted lines show the quantitative Elson-Morgan reaction.

These findings seem to confirm our earlier suggestion[1] that the mechanism of FU-induced "osmotic imbalance" involves a metabolic imbalance between cytoplasmic and cell wall syntheses. This condition is initiated by an early injury of the cell wall synthesizing mechanism by FU and is accompanied by relatively uninhibited cytoplasmic growth. As a result, after the removal of the FU from the medium the bacteria may resume a sort of unbalanced growth which soon leads to the disintegration of the cells.[8, 9]

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† Predoctoral Fellow of the National Institutes of Health, U. S. Public Health Service (EF-9085).

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[9] The 60 minutes' incubation time with FU—a standard condition used in our experiments—may represent the critical time up to which the above mechanism of action of the drug predominates. On prolonged incubations inhibitions of other phases of pyrimidine metabolism may become decisive.

ACTION SPECTRA OF CHROMATIC TRANSIENTS AND THE EMERSON EFFECT IN MARINE ALGAE

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Chromatic transients are the changes in oxygen evolution rate recorded on altering the color of light incident on a tissue, even though the intensities are adjusted to give equal steady rates of photosynthesis at both wavelengths. These were first observed[1] on exposing a red alga (*Porphyra*) alternately to red light (675 mμ) and green light (560 mμ). They have been reported[2] in a green alga (*Ulva*) on alternating illumination between (a) 490 and 540 mμ; and (b) 640 and 688 mμ. The location of these regions coincided fairly closely to the absorption *in vivo* of several pigments: chlorophyll *a* at 675–688 mμ; mixed chlorophylls at 540 mμ; chlorophyll *b* at 640–650 mμ; phycoerythrin at 560 mμ, and carotenoids at 490 mμ. (Alterations of wavelength within the absorption region of a single pigment produce little or no transient.)

The earlier studies were made by rapid change of setting on a monochromator, and the regions were limited by output energy and action spectra of the tissue to a few pairs of wavelengths. In order to obtain a more accurate delineation of the entire action spectrum for the transients, two sources of light have now been employed, one a fixed or reference wavelength, derived from a separate lamp with interference filter, the other the high-energy monochromator previously described.[3] The images of the two lamp filaments were focused carefully upon the tissue, usually an algal thallus one cell (or a few cells) thick, tightly held against a bright platinum electrode by means of a cellophane strip.[3] *Anabaena*, a blue green alga, was studied by lifting mats of filaments which had grown out very uniformly at the surface of sea water, and spreading them as smoothly as possible over the electrode.

Oxygen arriving at the electrode was reduced to H_2O_2 at an applied potential of 0.5 volt, the current being recorded by a Speedomax potentiometer connected across a fixed resistance (usually 1,000 ohms) in the circuit. Flowing or recirculated sea water, usually equilibrated against 5 per cent CO_2 in air, quickly established a base line which remained very steady in the dark, frequently at around

2 or 3 μamp, due to the passage of O_2 across the tissue. It was increased in the light to as much as 8 or 10 μamp, due to the arrival of photosynthetically produced O_2 at the electrode, the level being dependent upon light intensity. This is therefore read as a *rate* of photosynthesis and, by means of the electrochemical equivalent of H^+ ions oxidized, can be made an absolute measure of O_2 diffusing to the electrode. Furthermore, due to the short diffusion distance (a few microns only, the thickness of the cell wall), the new equilibrium value is quickly established, in a matter of one or 2 minutes (Fig. 1). This makes it possible to follow transients fairly sensitively.

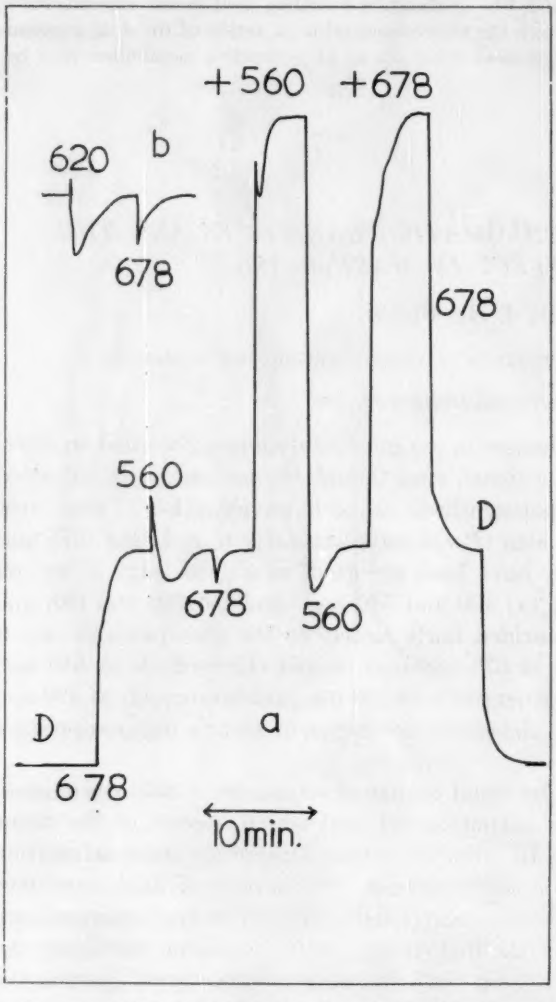


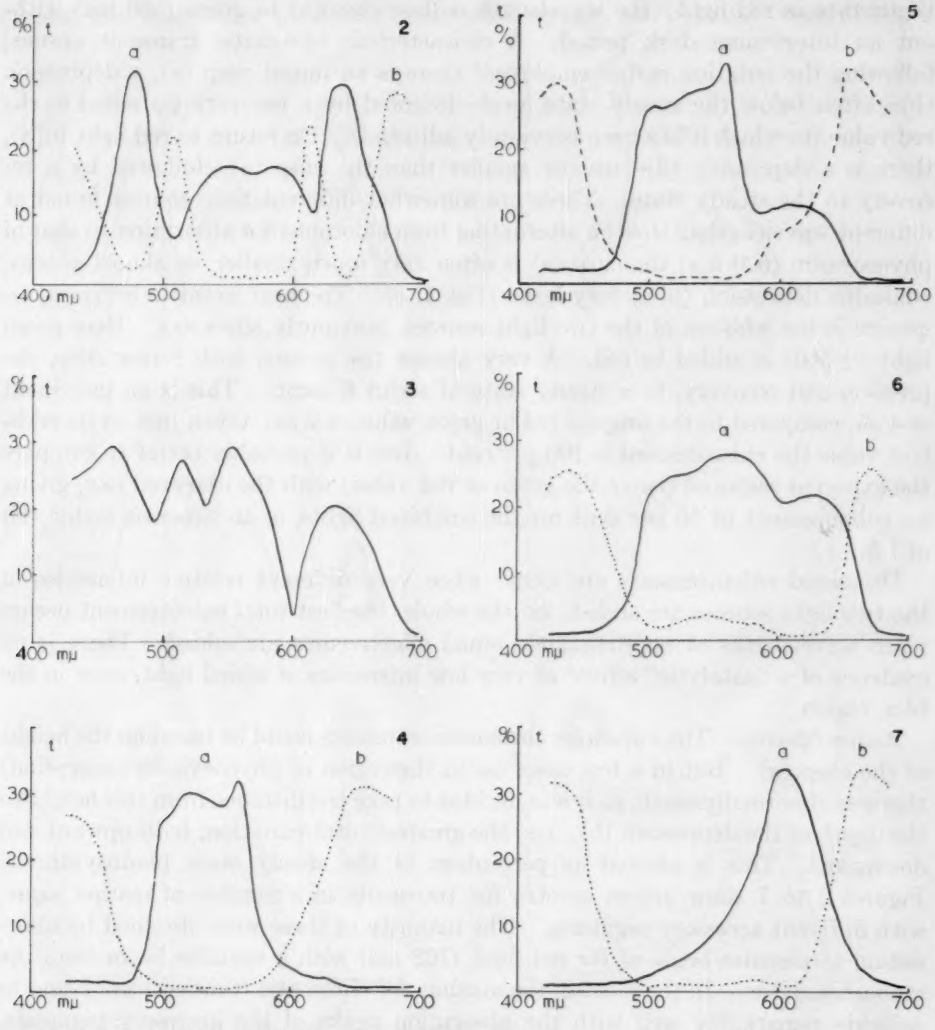
FIG. 1*a*.—Chromatic transients and the Emerson enhancement due to alternation and addition of red (678 mμ) and green light (560 mμ), in the red alga *Porphyra Nereocystis*. *b*.—Chromatic transients on alternation of 678 and 620 mμ. Explanation in text.

One precaution in recording chromatic transients is that the tissue (or the light beam) be very carefully restricted to the surface of the electrode. If the tissue projects beyond its edges, there may be a slow diffusion of O_2 from the sides to the electrode (despite good flow of sea water) which gives spurious transients if the beams of light do not exactly coincide. Careful adjustment of the beams and tissue to the electrode size or, in some cases, masking with black paper or black cellophane obviated this "edge effect" (which may be readily tested by alternating an identical wavelength from the two sources).

Another source of spurious chromatic transients is poor synchronization of the light shutters. While electrical switching partially obviated this trouble, it was found best to utilize a sliding shutter which very accurately interrupted one beam as it exposed the other. A check with photocells is desirable.

Emerson Effect.—This phenomenon, discovered by the late Robert Emerson,[4, 5] signifies the more than additive effect of two superposed wavelengths; thus the far red (700 mμ), which is usually inefficient in the photosynthesis of green plants,

becomes much more effective when shorter wavelengths are given simultaneously. This is ascribed to the low activity of chlorophyll *a*, which is markedly enhanced when light absorption by chlorophyll *b* or *c*, phycocyanin, phycoerythrin, fucoxanthin, or other carotenoids occurs simultaneously. Since two alternating light



Action spectra of chromatic transients (t):
FIG. 2.—*Enteromorpha tubulosa;* reference beam (*a*) 702 mμ; (*b*) 647 mμ. FIG. 3.—*Punctaria occidentalis;* reference beam 702 mμ. FIG. 4.—*Porphyra Thuretii;* reference beam (*a*) 702 mμ; (*b*) 566 mμ. FIG. 5.—*P. Nereocystis;* (same). FIG. 6.—*P. perforata;* reference beam (*a*) 702 mμ; (*b*) 614 mμ. FIG. 7.—*Anabaena* sp.; (same).

sources were found desirable to trace the transient action spectra, it was a simple matter to *combine* two wavelengths (ordinarily adjusted to give equal photosynthesis separately) and observe the enhancement. (Had such a double source been employed earlier, the enhancement would no doubt have been observed some time ago.)

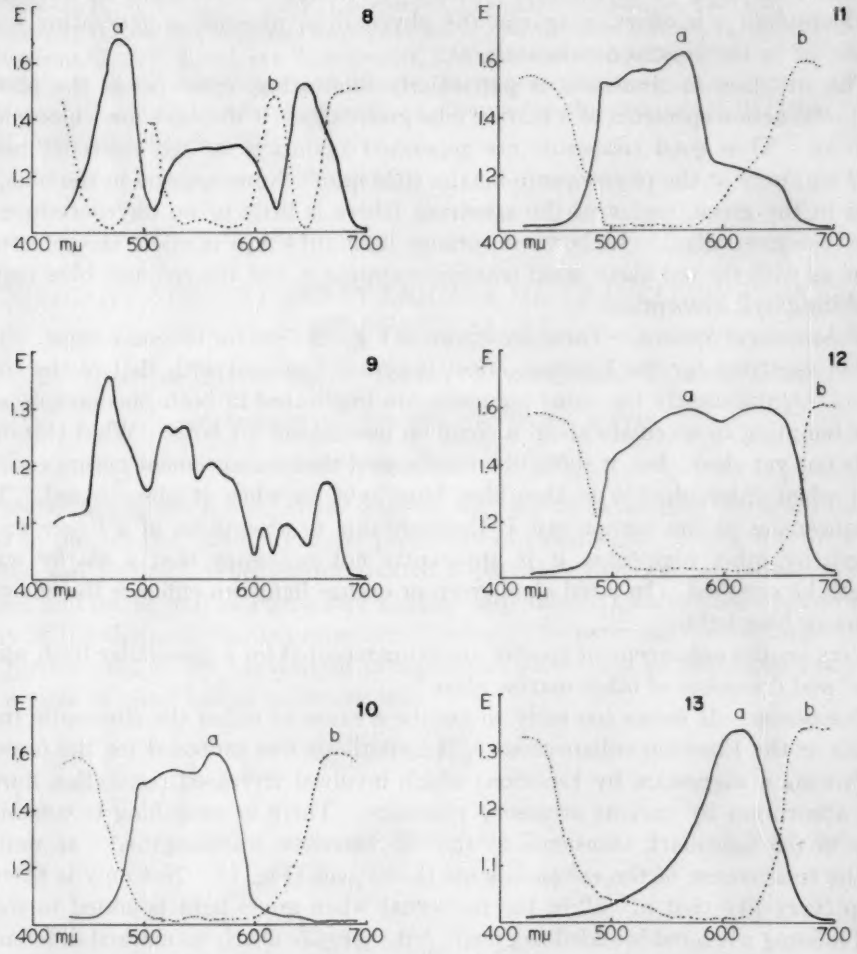
Recording.—Figure 1 shows a characteristic sequence of exposures in a red alga (*Porphyra Nereocystis*). The initial level of current (2 μamp) is due to oxygen arriving at the electrode across the tissue in the dark (D). Red light (678 mμ) near the absorption peak of chlorophyll *a* is then given; the polarographic current is increased to about twice the dark value. This represents the steady photosynthetic rate in red light; the wavelength is then changed to green (560 mμ) without an intervening dark period. A characteristic chromatic transient ensues; following the notation earlier employed[2] there is an initial cusp (a), a depression (b)—often below the steady state level—followed by a recovery (c) equal to the red value (to which it has been previously adjusted). On return to red light (678), there is a depression (d)—usually smaller than the cusp (a)—followed by a recovery to the steady state. There are somewhat different time courses found at different wavelengths; thus on alternating from chlorophyll *a* absorption to that of phycocyanin (620 mμ) the cusp (a) is often very much smaller, or almost absent, while the depression (b) is very large (Fig. 1, *b*). The next event in a typical sequence is the *addition* of the two light sources, previously *alternated.* Here green light (+560) is added to red. A very abrupt rise occurs, with a new cusp, depression and recovery, to a steady state of about 6 μamp. This is an increment of 4 μa, compared to the original red or green value of 2 μa; taken just on its additive value the enhancement is 100 per cent. But it is probably better to compare the expected response (twice the green or red value) with the observed one, giving an enhancement of 50 per cent for the combined lights, or an Emerson factor (E) of 1.5.

Decreased enhancements are found when very different relative intensities of the two light sources are added; on the whole, the best total enhancement occurs when wavelengths of approximately equal effectiveness are added. There is no evidence of a "catalytic" effect[6] at very low intensities of added light, even in the blue region.

Action Spectra.—The values for chromatic transients could be based on the height of the cusp (a). But in a few cases (as in the region of phycocyanin absorption) this was abnormally small, so it was decided to take the distance from this height to the depth of the depression (b); i.e., the greatest total variation, both upward and downward. This is plotted as percentage of the steady state photosynthesis. Figures 2 to 7 show action spectra for transients in a number of marine algae, with different accessory pigments. The majority of these were obtained by alternating a reference beam of far red light (702 mμ) with a variable beam from the monochromator. In these cases, the maxima for chromatic transients are found to coincide remarkably well with the absorption peaks of the accessory pigments. Thus in the green alga (*Enteromorpha*) the highest activity was found at 640–650 mμ (chlorophyll *b* absorption) and 480–490 mμ (carotenoid absorption). (The latter could conceivably be due partly to chlorophyll *b*, and the somewhat lower activity in the middle of the spectrum must also represent predominant absorption by chlorophyll *b*.) The peaks for the brown alga (*Punctaria*) must represent chlorophyll *c* (630 and 580 mμ), fucoxanthin (520–540 mμ), and other carotenoids (480 region); again, chlorophyll *c* might be contributing to the latter maximum.

The red algae, having no chlorophyll *b* or *c* (chlorophyll *d* seems to be nearly absent from most red algae as well), show little or no activity in the regions charac-

terizing such pigments; but they do display transients in the region 620–650 mμ depending upon the phycocyanin (and allophycocyanin) content. *Porphyra Thuretii* has the least of these pigments (it is a deep red color), and shows little or no transient activity until the phycoerythrin region is approached (wavelengths less than 600 mμ, with maximum at 560 mμ). *P. Nereocytsis* (a more purple species) has a shoulder indicating some phycocyanin activity (620 mμ), though



Action spectra for enhancement (Emerson effect, E):
FIG. 8.—*Enteromorpha*. FIG. 9.—*Punctaria*. FIG. 10.—*Porphyra Thuretii*.
FIG. 11.—*P. Nereocystis*. FIG. 12.—*P. perforata*. FIG. 13.—*Anabaena*.
(Wavelengths as in FIGS. 2–7 for the corresponding algae.)

again the major activity is at 560 mμ. Only in *P. perforata* (of a slate gray color) does the transient activity become very large in the phycocyanin region; here it extends well toward 650 mμ, corresponding to the high content of allophycocyanin in this species. It should be noted that the transient activity is very slight in the blue end of the spectrum (400–500 mμ) for all these species; quite clearly, the carotenoids are unable to generate transients against the far red (nor, obviously,

can chlorophyll *a* in this region of its absorption). However, as Figure 4 indicates, the blue end of the spectrum is perfectly capable of generating good transients when alternated with green (or orange) light absorbed by the *phycobilins*. This is clearly shown in several of the figures, where a reference beam of 566 or 614 mμ was employed. Against such a reference beam good activity is shown in *both* ends of the spectrum (the intensity of blue light has to be very high, due to its low photosynthetic efficiency in red algae).[3] It must be stressed that *either* absorption region of chlorophyll *a* is effective against the phycobilins, whether in generating transients, or in the Emerson enhancement.

The situation in *Anabaena* is particularly interesting, since (as in the photosynthetic action spectrum of a marine blue green alga[3]) it displays low chlorophyll activity. Thus good transients are generated against a far red reference beam (702 mμ) only at the phycocyanin maxim (620 mμ). None appears in the blue, or even in the green, region of the spectrum (there is little or no phycoerythrin in this blue-green alga). If, however, orange light (614 mμ) is made the reference then, as with the red algae, good transients appear in *both* the red and blue region of chlorophyll absorption.

Enhancement Spectra.—These are shown in Figures 7–12 for the same algae. The action spectrum for the Emerson effect is almost identical with that of the transients. Quite clearly the same pigments are implicated in both phenomena, and it is tempting to speculate about a common mechanism for both. What this may be is not yet clear; but it should be emphasized that enhancement occurs equally well when chlorophyll *a* is absorbing blue light as when it absorbs red. The enhancement in the former case is therefore due to absorption of a *longer* wavelength by other pigments; it is apparently not necessary that a *shorter* wave length be supplied. In a red alga, green or orange light can enhance the effect of violet or blue light.

Very similar enhancement spectra are being reported for a unicellular fresh water alga[7] and a number of other marine algae.[8]

Discussion.—It seems too early to ascribe a cause to either the chromatic transients or the Emerson enhancement. A hypothesis was proposed for the former[2] (following a suggestion by Emerson) which involved increased respiration during the absorption by various accessory pigments. There is something to commend this in the light-dark transients at the characteristic wavelengths,[1, 2] as well as in the time course of the enhancements themselves (Fig. 1). Not only is there a cusp (very like that of "a" in the transient) when green light is added to red—and missing when red is added to green; but there are nearly as marked differences when the supplementary light is removed. On turning off the red light (leaving only green), there is a marked depression (enhanced respiration?), while this is not observed when green is turned off (leaving only red). Experiments at higher and lower temperature, and with a variety of respiratory and photosynthetic inhibitors, are under way to clarify these effects.

Summary.—Action spectra are presented for chromatic transients and the Emerson effect ("enhancement") in the photosynthesis of a number of marine algae. When far red light (700 mμ) is altered or supplemented with other wavelengths, the greatest effects occur at the absorption maxima of the accessory pigments: chlorophyll *b* and carotenoids in green algae; chlorophyll *c* and fucoxan-

thin in brown algae; phycobilins in red and blue-green algae. Both effects also occur equally well when the chlorophyll *a* is absorbing in the blue end of the spectrum (435 mμ).

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SIGNIFICANT STRUCTURES IN LIQUIDS, III. PARTITION FUNCTION FOR FUSED SALTS

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Very little has been done in the past in the way of developing partition functions to predict the thermodynamic properties of fused salts. Using the work of Walter and Eyring,[1] Wheeler[2] formulated a partition function for molten alkali halides and he, as well as Parlin and Eyring (unpublished), calculated successfully many of the thermodynamic properties of salts. The partition function used here is a further step in the application of significant structures as developed earlier.[3] For a mole of alkali halide molecules it is

$$f = \left\{ \frac{e^{\frac{E_s}{2RT}\left(\frac{V}{V_s}\right)^{1/3}}}{(1 - e^{-\theta/T})^3} \left[1 + n_h \cdot e^{\frac{-aE_s(V/V_s)^{1/3}}{2n_hRT}} \right] \right\}^{\frac{2V_s}{V}N} \times \left\{ \frac{(2\pi mkT)^{3/2}eV}{Nh^3} \frac{8\pi^2 IkT}{h^2} \frac{1}{(1 - e^{-h\nu/kT})} \right\}^{\left(1 - \frac{V_s}{V}\right)N} \quad (1)$$

$$n_h = n\left(\frac{V}{V_s} - 1\right) \quad (2)$$

The various parameters are explained as follows: E_s is the potential energy of the solid at the melting point; θ is the Einstein temperature of the solid; V_s is the volume of the solid per mole at the melting point; V is the molar volume of the liquid; n and a are parameters to be fitted to the experimental liquid data; m, I, and ν are the mass, the moment of inertia, and the ground state vibrational frequency, respectively, of the gaseous diatomic alkali halide molecule; T is the absolute temperature, R the gas constant, N Avogadro's number, k is Boltzmann's constant, and h is Planck's constant.

The two significant structures described by the partition function are solid-like and gaslike. In the solid-like structure as far as the partition function is concerned, the alkali metal ions are assumed to be equivalent to the halide ions. In other words, the alkali metal and halide ions are considered to be vibrating with a common Einstein frequency. The solid-like portion of the partition function is raised to the power $2(V_s/V)\ N$ because a mole of alkali halide crystal contains $2N$ particles when all of the ions are assumed to be equivalent. Also E_s/N is the potential energy per alkali halide molecule. Since the desired potential energy is the potential energy per ion, E_s must be divided by 2. The gaslike significant structure is composed of diatomic alkali halide molecules whose moments of inertia and ground state vibrational frequencies are assumed to be the same as for the vapor phase. To account for the long range nature of the coulombic interionic potential, the potential energy for the solid E_s has been multiplied by the factor $(V/V_s)^{1/3}$.

Calculations of melting point and boiling point data and critical constants have been carried out for four alkali halides, NaCl, KCl, NaBr, and KBr. The method of calculation was the same as that of Eyring, Ree, and Hirai.[3]

The parameters E_s and θ for the solid can be determined by various techniques. The value of θ may be calculated from the equation $\theta = 3\theta_D/4$ where θ_D is the

TABLE 1

	NaCl	KCl	NaBr	KBr
V_s, cc	30.19[4, *]	41.57 cc[4]	36.02 cc[4]	48.06 cc[4]
E_s, kcal/mole	56.07[5]	54.15[5]	51.91[6]	51.53[6]
θ, °K	215[7]	170[7]	145[8]	118[8]
I(gm cm^2)	1.291×10^{-38}[8]	2.195×10^{-38}[8]	1.856×10^{-38}[8]	3.468×10^{-38}[8]
$\omega = \nu/c$, cm^{-1}	380[9]	305[9]	302[10]	231[9]
n	9	6	7	5.5
a	0.1000	0.03000	0.05300	0.02300

* The reference numbers for experimental quantities are listed in the table.

Debye characteristic temperature determined experimentally at low temperatures. With θ known a value for E_s is chosen in the solid partition function to give an observed property of the solid at the melting point such as the vapor pressure or the energy of sublimation. Alternatively since the Debye temperature of a solid is usually lower at the melting point than for low temperatures, two independent experimentally determined parameters of the solid at the melting point can be used to find both E_s and θ.

For NaCl and KCl θ and E_s were determined by the first method. That is, three fourths of the experimentally determined θ_D was used for θ, and then the vapor pressure of the solid at the melting point was used along with the already determined θ to find E_s. For NaBr and KBr θ and E_s were determined by using the experimentally determined energy of sublimation and the vapor pressure at the melting point. The results obtained by the two methods will in general be slightly different but lead to about equally good values for the liquid properties. This is true since the parameters n and a in the liquid partition function can be varied to offset small deviations in the values of E_s and θ.

The values used for the various parameters are given in Table 1.

All of the properties calculated for the salts are listed in Tables 2, 3, and 4. As one can observe, the melting temperature was predicted for NaCl and KCl, while

for NaBr and KBr the melting temperature was fitted exactly by adjusting the parameters n and a.

In Table 2, T_m, V_m, and ΔS_f are the melting temperature, volume of the liquid, and the entropy of fusion, respectively. In Tables 3 and 4 these same properties carry the subscripts B and c to indicate the boiling point and the critical point respectively. References to experimental quantities are again listed by the particular value. The critical constants are, of course, not known experimentally.

Thus, the agreement with the observed data for all of the salts investigated is reasonably good, and the order of magnitude of n is what might be expected from previous work. There are one or two points which need to be discussed, however. First, as can be seen from recent publications,[4, 11] the experimental value of V_s is uncertain. Moreover, a recent publication[12] indicates that the vapors of the alkali halides are strongly associated to four atom and even six atom complexes. In the present treatment only diatomic molecules were assumed to constitute the

TABLE 2

MELTING-POINT PROPERTIES OF THE ALKALI HALIDES

		NaCl	KCl	NaBr	KBr
T_m, °K	Calc	1,070	1,023	(1,023)	(1,008)
	Obs	1,074[6]	1,049[6]	1,023[6]	1,008[6]
V_m, cc	Calc	38.14	49.06	43.84	56.13
	Obs	*37.74[4]	48.80[4]	44.08[4]	56.03[4]
ΔS_f, eu	Calc	7.75	5.40	6.16	4.83
	Obs	6.3[6]	5.8[6]	5.96[6]	6.94[6]

TABLE 3

BOILING-POINT PROPERTIES OF THE ALKALI HALIDES

		NaCl	KCl	NaBr	KBr
T_B, °K	Calc	1,750	1,684	1,671	1661
	Obs	1,738[6]	1,680[6]	1,665[6]	1653[6]
V_B, cc	Calc	51.15	71.20	60.15	81.81
	Obs	Unreported	Unreported	Unreported	Unreported
ΔS_v, eu	Calc	21.55	21.63	21.02	20.89
	Obs	23.5[6]	23.1[6]	23.2[6]	22.4[6]

TABLE 4

CRITICAL CONSTANTS OF THE ALKALI HALIDES

	NaCl	KCl	NaBr	KBr
T_c, °K	3,600	3,092	3,364	3,060
V_c, cc	293	431	342	482
P_c, atm	235.5	135.5	186.1	118.3

gaslike significant structure. Taking account of polymers of the salt molecules in the gaseous part of the partition function requires no new parameters but should improve the partition function.

Recent literature also has given new experimental values for the ground state vibrational frequencies of the diatomic alkali halides.[10] In this case, however, it has been verified that the new values affect the calculated results but slightly. In conclusion it can be said that although some significant structures have been neglected and uncertainties in the values of some experimental parameters exist, nevertheless the results prove that the general method of approach can be very fruitful and readily applied to calculate the thermodynamic properties of salts.

Very recently, Blomgren[13] applied the significant structure theory of liquids to molten KCl in a slightly different manner from our approach. But he obtained

reasonable agreement with experiment for the molar volume. An extensive review was made for fused salts by Blomgren and Van Artsdalen.[14]

The authors wish to thank the National Science Foundation and the Office of Ordnance Research, United States Army for support of this work.

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*SIGNIFICANT STRUCTURES IN LIQUIDS, IV. LIQUID CHLORINE**

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Earlier investigators[1, 2] attained considerable success in fitting the liquid state through the use of the "holes-in-solid" model. Extending this theory, Eyring and co-workers[3, 4] have recently proposed the theory of significant structures in liquids and have thus obtained a relatively simple and accurate partition function for normal liquids such as neon, argon, krypton, xenon, benzene, methane and nitrogen, and also for fused salts and molten metals.

According to the significant structure theory, a liquid molecule possesses both solid-like and gaslike degrees of freedom. The relative contribution of the two types is given by V_s/V and $(V - V_s)/V$, respectively, where V_s is the molar volume of the solid at the melting point and V is the molar volume of the liquid at various temperatures. Thus, the partition function for the liquid is given by

$$f_L = (f_s)^{NV_s/V}(f_g)^{N(V-V_s)/V} \tag{1}$$

where f_s and f_g are the partition functions for the solid-like and gaslike parts.

The halogens seemed a particularly interesting group of elements on which to apply the theory, and chlorine was chosen to be the first of the halogen group because of its general interest and the availability of ample data against which calculated values might be checked.

Partition Function for Liquid Chlorine.—In writing the various terms of the respective partition functions of the solid-like and gaslike parts, certain assumptions had to be made concerning the degrees of freedom of the chlorine molecule in these two states. The unusually high entropy of fusion reported by Giauque and Powell[5] and the absence of transition peaks in the heat capacity curve for solid chlorine given by the latter indicates that the chlorine molecule probably does not rotate freely in the solid state. Also the density data on liquid chlorine[6, 7] suggests that rotation is inhibited in the liquid state. This question is discussed further below.

Rather than complicate the partition function by considering libration-type degrees of freedom, the solid-like part was treated as a five-degree Einstein oscillator, with the internal vibration being the sixth degree of freedom. Free rotation was assumed in the gaslike part of the partition function.

The resulting partition function for the liquid, then, assumed the following form:

$$f_L = \left\{\frac{e^{E_s/RT}}{(1-e^{-\theta/T})^5}\,\frac{1}{1-e^{-h\nu/kT}}\,(1+n_h e^{-a'/n_h RT})\right\}^{NV_s/V} \left\{\frac{(2\pi mkT)^{3/2}}{h^3}\,\frac{8\pi^2 IkT}{2h^2}\,\frac{1}{1-e^{-h\nu/kT}}\,\frac{eV}{N}\right\}^{N(V-V_s)/V} \quad (2)$$

Equation (2) contains two parameters which have the following connotations. n_h represents the number of "holes" or equilibrium sites accessible to a molecule in addition to its single most stable position. This could also be considered to be the moles of holes per mole of normal sites. The latter description can be equated to the total volume available for holes divided by the volume per mole of holes, or

$$n_h = \frac{(V-V_s)}{(V_s/n)} \equiv n(x-1) \quad (3)$$

where n is the number of holes whose combined volumes equal the volume of one molecule, or $(1/n)$ is the fraction of the volume of a single molecule required per new equilibrium site. The term x is a reduced volume used to express the ratio (V/V_s).

The second parameter a' represents the energy put into the system by expansion, and is related to the heat of sublimation E_s by the expression

$$a' = a''E_s \quad (4)$$

The strain energy, or energy contributed per hole, E_h, then, is $a''E_s/n_h$. Substituting the value for n_h from equation (3), we then obtain,

$$E_h = \frac{a''E_s}{n(x-1)} = aE_s/(x-1) \quad (5)$$

The degeneracy term, then, becomes

$$1 + n(x-1)\,e^{-aE_s/RT(x-1)}$$

and the partition function takes its final form, which now includes the two new parameters n and a:

$$f_L = \left\{\frac{e^{E_s/RT}}{(1-e^{-\theta/T})^5}\,\frac{1}{1-e^{-h\nu/kT}}\,[1+n(x-1)e^{-aE_s/RT(x-1)}]\right\}^{N/x}$$
$$\left\{\frac{(2\pi mkT)^{3/2}}{h^3}\,\frac{8\pi^2IkT}{2h^2}\,\frac{1}{1-\epsilon^{-h\nu/kt}}\,\frac{eV_s}{N}\,x\right\}^{N(1-1/x)} \quad (6)$$

The Helmholtz free energy A is perhaps the most useful function related to the partition function, as it is readily converted into the other thermodynamic functions. Since it involves the logarithm of the partition function, it was found convenient to adopt the following abbreviations for component terms:

$$A = -\frac{RT}{x}(\sigma_1+\sigma_2) + RT\left(1-\frac{1}{x}\right)(\gamma+\ln x) \quad (7)$$

where,
$$\sigma_1 \equiv \frac{E_s}{RT} - 5\ln(1-e^{-\theta/T}) - \ln(1-e^{-h\nu/kT}) \quad (8a)$$

$$\sigma_2 \equiv \ln\left\{1+n(x-1)\exp\left[\frac{-aE_s}{RT(x-1)}\right]\right\} \quad (8b)$$

$$\gamma \equiv \ln\left[\frac{(2\pi mkT)^{3/2}}{h^3}\,\frac{8\pi^2IkT}{2h^2}\,\frac{1}{1-e^{-h\nu/kT}}\,\frac{eV_s}{N}\right] \quad (8c)$$

It will be noted that σ_1 and γ are free of any x terms, and hence can be treated as constants, if T is kept constant.

The observed $x_m = 1.198$ ($V_s = 34.341$, $V = 41.145$) at the melting point (171.4°K) is abnormally high, since most solids increase about 10 per cent upon melting. This value of x_m, however, is probably correct, although a small uncertainty exists due to the fact that density data for both phases had to be extrapolated to the melting point. The anomality for chlorine is probably due to some transition in the solid beginning at a temperature near the melting point. Thus, we had to choose a molar volume for the solid state larger than that observed and more of the order of what it might be for the complete transition mentioned above. By a trial-and-error method, we obtained $x_m = 1.05$.

Equation (6) was solved for σ_1 and σ_2 for $x_m = 1.05$ ($V_s = 39.1757$ cc/mole), according to the method developed by Fuller[8] in fitting benzene. This yielded a value of 14.3 for n, a θ-value of 41.48°K, an E_s value of −6,075.9 cal/mole and an a-value equal to 0.0012977.

Results.—(*a*) *Properties at the boiling and the critical points:* The equation was tested at the boiling point (67 degrees above the melting point) and yielded the following results, listed and compared with observed values in Table 1:

TABLE 1

BOILING-POINT DATA FOR CHLORINE

	Calc.	Obs.	Error, %
Liquid volume, cc/mole	44.856	45.321	−1.03
Vapor pressure, atm.	0.9984	1.0000	−0.161
Temperature, °K	(239.05)	239.05	...
Entropy of Vaporiz'n, e.u.	20.46	20.40	0.27

Critical point properties were calculated by setting the first and second derivatives of P with respect to V equal to zero, P being equal to $-(\partial A/\partial V)_T$. The

results are shown in Table 2. The relatively larger error compared to similar calculations previously reported for the inert gases[3, 4] is somewhat to be expected considering the longer liquid range (245 degrees) of chlorine. The error is large enough, however, to conclude that the theory still needs some modification to take care of the critical point.

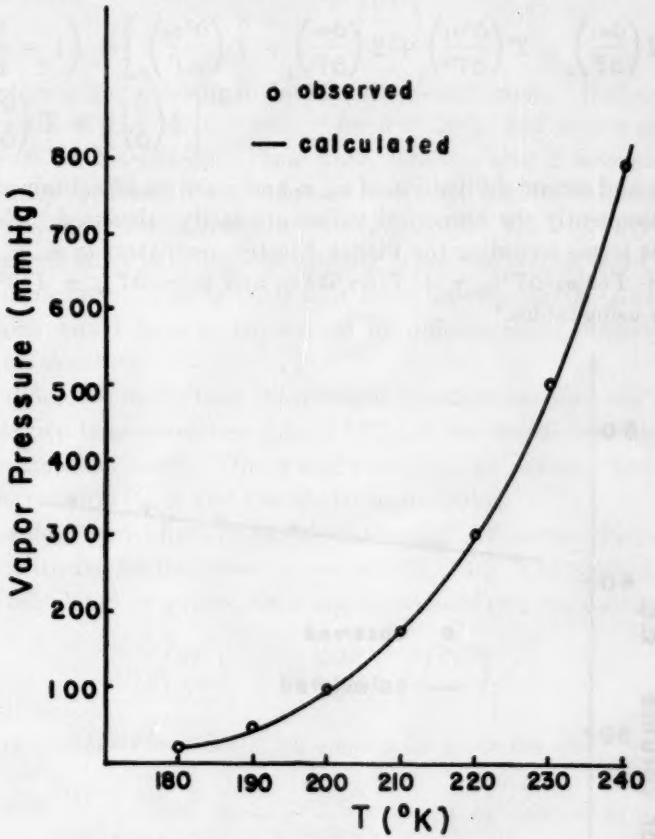

FIG. 1.—Vapor pressure versus temperature.

(*b*) *Vapor pressures and molar volumes:* A further check on the accuracy of the function was made for temperatures between the melting and boiling points of chlorine. Figure 1 shows a comparison of calculated and observed values for the vapor pressure over the liquid range indicated. The observed data is from the

TABLE 2

CRITICAL POINT PROPERTIES FOR CHLORINE

	Calc.	Obs.	Error, %
Critical temperature, °K	463.5	417.16	11.1
Critical volume, cc/mole	131.2	123.8	5.98
Critical pressure, atm.	104.74	76.1	37.7

work of Giauque and Powell.[5] In Figure 2 calculated molar volumes are compared with those observed by Johnson and McIntosh[7] over the range from the melting point to the boiling point.

(c) *Heat capacity:* The heat capacity of liquid chlorine was calculated by differentiating the expansion for the liquid entropy, equation (9), with respect to temperature at constant volume, and multiplying the derivative by T, i.e.,

$$S = \frac{R}{x}\left[\sigma_1 + T\left(\frac{\partial\sigma_1}{\partial T}\right)_x + \sigma_2 + T\left(\frac{\partial\sigma_2}{\partial T}\right)_x\right] + R\left(1 - \frac{1}{x}\right)\left[\gamma + T\left(\frac{\partial\gamma}{\partial T}\right)_x + \ln x\right] \quad (9)$$

$$C_V = \frac{RT}{x}\left[2\left(\frac{\partial\sigma_1}{\partial T}\right)_x + T\left(\frac{\partial^2\sigma_1}{\partial T^2}\right)_x + 2\left(\frac{\partial\sigma_2}{\partial T}\right)_x + T\left(\frac{\partial^2\sigma_2}{\partial T^2}\right)_x\right] + \left(1 - \frac{1}{x}\right)\left[2\left(\frac{\partial\gamma}{\partial T}\right)_x + T\left(\frac{\partial^2\gamma}{\partial T^2}\right)_x\right] \quad (10)$$

Here the first and second derivatives of σ_1, σ_2 and γ are readily obtained from equation (8); consequently the numerical values are easily calculated. Especially the values for the terms including the Planck-Einstein oscillators in $\sigma_1 + T(\partial\sigma_1/\partial T)_x$, $2(\partial\sigma_1/\partial T)_x + T(\partial^2\sigma_1/\partial T^2)_x$, $\gamma + T(\partial\gamma/\partial T)_x$, and $2(\partial\gamma/\partial T)_x + T(\partial^2\gamma/\partial T^2)_x$ are facilitated by using tables.[9]

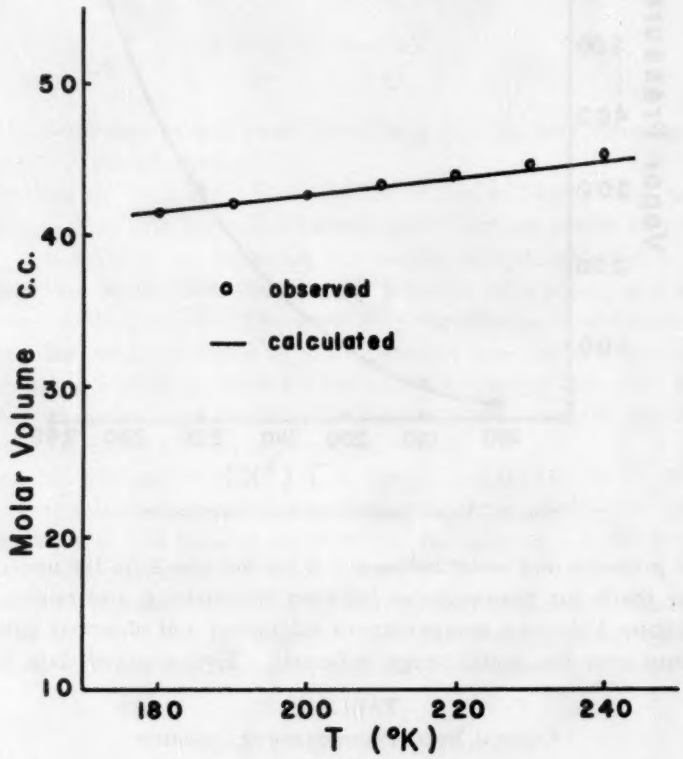

FIG. 2.—Molar volume versus temperature.

Since the partition function yields C_V rather than C_p, it was converted to the latter through the use of equation (11):

$$C_p - C_V = \frac{TV\alpha^2}{\beta} \quad (11)$$

Here α is the coefficient of thermal expansion for liquid chlorine, and β is its compressibility. These two coefficients are as follows:

$$\alpha = \frac{1}{V}\left(\frac{\partial V}{\partial T}\right)_p \tag{12}$$

$$\beta = -\frac{1}{V}\left(\frac{\partial V}{\partial P}\right)_T \tag{13}$$

The literature is quite lacking in data for these coefficients. Richards and Stull[10] reported a value of 118×10^{-6} atm.$^{-1}$ for β at 20°C and over a pressure range from 9.9 to 98.7 atmospheres. Their work indicates that β decreases with pressure. Generally speaking, it also increases with temperature, although this is more directly due to the effect of temperature on density. The reported figures could hardly be extrapolated to conditions existing below the boiling point without considerable uncertainty. This is true, to a lesser extent, for the coefficient of expansion, which would have to be derived by differentiating empirical equations given for liquid densities.

Since the difference in the heat capacities at constant pressure and constant volume amounts to a large correction (about 35%), it was decided to calculate it from the partition function itself. This would constitute an extreme test of the function, since the errors in C_V, α, and β would be compounded.

The values for β were obtained by calculating $(\partial P/\partial V)_T$ from the partition function and substituting its reciprocal into equation (13). The coefficient of expansion, α, was calculated by a more circuitous route involving the following equation:

$$\left(\frac{\partial V}{\partial T}\right)_P = -\left(\frac{\partial P}{\partial T}\right)_V \Big/ \left(\frac{\partial P}{\partial V}\right)_T \tag{14}$$

The necessary equations for calculating α and β are given below:

$$P = \frac{RT}{V_s x^2}\left[-\sigma_1 - \sigma_2 + x\left(\frac{\partial \sigma_2}{\partial x}\right)_T + \gamma + (x-1) + \ln x\right] \tag{15}$$

$$\left(\frac{\partial P}{\partial T}\right)_V = \frac{R}{V_s x^2}\left\{-\left[\sigma_1 + T\left(\frac{\partial \sigma_1}{\partial T}\right)_z\right] - \left[\sigma_2 + T\left(\frac{\partial \sigma_2}{\partial T}\right)_z\right] + x\left(\frac{\partial \sigma_2}{\partial x}\right)_T + xT\left[\frac{\partial}{\partial T}\left(\frac{\partial \sigma_2}{\partial x}\right)_T\right]_x + \gamma + T\left(\frac{\partial \gamma}{\partial T}\right)_z + (x-1) + \ln x\right\} \tag{16}$$

$$\left(\frac{\partial P}{\partial V}\right)_T = \frac{RT}{V_s^2 x^3}\left[x^2\left(\frac{\partial^2 \sigma_2}{\partial x^2}\right)_T - 2x\left(\frac{\partial \sigma_2}{\partial x}\right)_T + 2\sigma_2 - x + 3 - 2(\gamma + \ln x - \sigma_1)\right] \tag{17}$$

The C_p data thus obtained is entirely theoretical and calculated from the partition function. Table 3 gives the results of this calculation and compares C_p with the observed values from Giauque and Powell.[5]

TABLE 3

CALCULATED VALUES FOR α, β, C_V, AND C_p FOR LIQUID CHLORINE AND OBSERVED VALUES FOR C_p

Temp., °K	$\alpha \times 10^3$	$\beta \times 10^6$	C_V	C_p	C_p (obs.)	Error, %
180.000	1.301	47.4	10.12	16.59	16.02	3.56
190.513	1.219	50.0	10.03	15.81	15.99	−1.13
200.413	1.216	55.3	10.13	15.67	15.95	−1.76
210.000	1.241	62.1	10.11	15.56	15.89	−1.45
219.909	1.254	68.3	10.11	15.47	15.84	−2.34
229.958	1.304	82.2	10.11	15.47	15.77	−1.90
240.050	1.356	88.9	10.11	15.51	15.70	−1.21

Discussion.—In view of the assumptions made concerning degrees of freedom in writing the partition function, it seems appropriate to now consider various ways in which chlorine molecules can pack in the solid and liquid states. Once a pattern is established, theoretical molar volumes can be calculated by inscribing whatever particular-shaped volume the molecule describes in its modes of motion inside a polyhedral-shaped figure that packs without voids. These values, then, can be compared with the observed molar volumes of the solid and the liquid at various temperatures, in order to get some idea about what might be happening.

The molar value for solid chlorine, 34.34 cc, was estimated from the figures given by Sapper and Biltz,[11] who extrapolated the density obtained by Heuse[12] at liquid nitrogen temperatures to absolute zero. This datum has recently been confirmed by X-ray studies of solid chlorine by Keesom and Taconis,[13] and by Collin.[14] The latter finds solid chlorine to be orthorhombic instead of tetragonal, as reported by the former investigators. Smyth[15] concludes, from the specific heat data of Eucken and Karwat,[16] that rotation of chlorine molecules is not possible in the solid state, in agreement with calculations given below.

Using Collin's figures of 2.02 Å and 1.67 Å for the bond distance and the van der Waals radius of chlorine, respectively, a molar volume of 31.19 cc is obtained for the solid from the formula, $V = 2\sqrt{3}r^2 lN$, assuming the structure to consist of hexagonally-packed hexagonal cylinders. Here, $r(= 1.67$ Å) is the radius of a circular cylinder contacting the inner surface of the hexagonal cylinder, l ($= 2.02 + 2 \times 1.67$) is the length of the latter, and N is the Avogadro number. Pauling's[17] figures for the bond distance (1.98 Å) and for the van der Waals radius (1.80 Å) give a value of 37.6 cc. Both agree roughly with the observed value, provided the molecules in the solid state pack as mentioned above.

For a minimum volume corresponding to free rotation for *all* the molecules, we can assume liquid chlorine to consist of face-centered, cubically close-packed spheres of 2.68 Å radius. This calls for a molar volume of $4\sqrt{2}r^3N$ ($=65.51$ cc), considerably above the volume of liquid chlorine, even at its boiling point ($V =$ 45.321 cc).

If we consider the arrangement where the molecule executes two-dimensional rotation, thus forming oblate ellipsoids rather than spheres, and if we inscribe each ellipsoid in a hexagonal title-shaped volume that packs without voids, we obtain a molar volume of $2\sqrt{3}\ r^2DN$ ($=50.2$ cc). Here $r = 2.68$ Å and D is the depth of the tile which equals twice 1.67 Å. This molar volume compares favorably with the value of 45.399 cc observed at the boiling point.

One must conclude, then, that chlorine cannot rotate appreciably in the solid state, and that only some of the molecules can rotate two-dimensionally at a given

time in the liquid state. The high entropy of fusion cannot be attributed to a general onset of rotation at the melting point, but rather would seem to indicate considerable association of the chlorine molecules with one another in the solid state. During its long, 245-degree liquid range chlorine does expand considerably and reaches a molar volume of 123.76 cc at the critical point, where rotation is definitely possible. Mellor[18] lists liquid chlorine as having the largest compressibility of all the elements at 20°C.

Summary.—A simplified partition function for liquid chlorine had been developed through the "holes-in-solid" model in accordance with the theory of significant structures. The function is made to fit only at the melting point and gives excellent agreement with observed data for vapor pressures, molar volumes, and C_p from the melting point to the boiling point, over a 67-degree range. Good agreement is also obtained for the entropies of fusion and vaporization, and calculated values of compressibility and coefficient of expansion are of the right magnitude.

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THE GENIC CONTROL OF MATING TYPES IN PARAMECIUM BURSARIA*

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Jennings'[1] discovery of a system of multiple mating types in *Paramecium bursaria* in 1938 followed closely Sonneborn's[2] initial description of mating types in *P. aurelia*. The detailed and brilliant experimental analyses carried out by Jennings[3] and directed toward an understanding of mating type inheritance and determination in this organism did not result in a satisfactory explanation for the various phenomena. The new investigations to be reported here, however, permit a resolution of some of the major difficulties. In particular, they demonstrate that the four complementary mating types long recognized for variety 1 are determined by specific combinations of complementary genes at two unlinked loci. Cells whose macronuclei carry dominant genes at both loci are mating type A; mating type C is brought about by the combination of homozygous recessives at both loci. The two alternative combinations of homozygous recessive alleles at one locus and at least one dominant gene at the other determine in a specific way mating types B and D.

Jennings considered a similar two-gene hypothesis untenable because it failed to account for certain of his observations; but since the new and more extensive results to be reported here support the hypothesis without ambiguity, an attempt will be made to reconcile, at least formally, the apparent exceptions originally discovered and reported by Jennings.

Since in ciliates the micro- and macronuclei of a clone are formed from mitotic products of a single initial nucleus, it can be inferred that they are isogenic. It has also been established that the micronuclei alone furnish the nuclear apparatus for new clones and that the mating type expressed by a cell is ultimately governed by its macronucleus. Within this framework, two systems of mating type control have been recognized. In *Euplotes patella*[4] and *Tetrahymena pyriformis* (variety 8),[5] (and perhaps *Paramecium caudatum*),[6] conventional genetic analyses reveal that specific mating types are determined by the genes brought together at the origin of the clone; thus, cells with a particular genotype express a predictable phenotype. On the other hand, in *P. aurelia*,[2] *P. multimicronucleatum*,[7] and *T. pyriformis* (variety 1)[8] macronuclei with presumably identical genotypes are characteristically found to govern the expression of different mating types; a single clone commonly contains cells expressing complementary types. Here a given mating type is not determined by a unique genotype *per se*, but instead the genetically pluripotent macronucleus is induced to express only one of the two (or more) possible types. The mechanism of nuclear induction or "mutation" and final mating type expression have been studied by Sonneborn[9] and Nanney.[8]

Establishment of the two-gene hypothesis for mating type determination in *P. bursaria* aligns this organism with *Euplotes* and *T. pyriformis* (variety 8); as a rule each mating type reflects a specific genic combination. However, in certain exceptional clones, Jennings reported mating type instability ("self-differentiation"),

i.e., the production, within a sexually mature clone, of cells expressing a new mating type. Moreover, qualitatively unexpected mating types appear infrequently among the progeny of various crosses. These facts suggest that "nuclear instability," similar to that in newly developed macronuclei in *P. aurelia* and *T. pyriformis* (variety 1), may occur rarely in *P. bursaria*. If so, the mechanism of mating type determination in *P. bursaria* would appear to combine elements of the two major classes of determinative systems in ciliates. A similar evaluation of the system in *T. pyriformis* (variety 8) has been set forth recently by Orias.[5]

Materials and Methods.—The taxonomic species *Paramecium bursaria* includes a large number of clones collected from nature and their sexual progeny. Jennings showed that each clone is normally self-sterile and that fertile interclonal conjugations occur only according to a recognized pattern. Interfertile clones belong to a common variety; six reproductively isolated varieties are presently known.

The strains studied by Jennings are no longer available. Four clones were isolated from Malibu Lake, California, and each was identified as representative of one of the four standard mating types known for variety 1.[10] It was important to establish at the outset that these clones conform in a general way to those previously employed. Comparisons of the two groups of clones with respect to the (1) cytology of sexual and asexual reproduction, (2) the diurnal periodicity of sexual activity in mature cells, (3) the "life cycle" stages, and (4) the deleterious effects of inbreeding as opposed to outcrossing, gave no indication of important differences.

Culture methods for *P. bursaria* have been described[3, 11] and follow closely those for *P. aurelia*.[12] Exconjugant clones were maintained in serial isolations permitting maximal fission rates, and were found to attain sexual maturity 50 to 100 fissions after their origin. Mass cultures of mature and sexually reactive cells were made available by transferring exconjugant clones to test tubes, thereafter kept at 25°C and afforded diurnal periods of light. Samples of each exconjugant clone were mixed with samples of each of the four standard mating types in order to discover the mating type of the clone. The unknown clone was classified as a particular type if it failed to mate with the standard of that type and formed pairs with the remaining three standard types. The precautions described by Jennings for avoiding abortive conjugations in selecting pairs for genetic analysis were followed throughout.

Cytogenetics.—Chen[13] has provided the cytological details of nuclear reorganization during conjugation in *P. bursaria*. The micronucleus in each animal of the mating pair undergoes two maturation (meiotic) divisions, one daughter nucleus disintegrating after each division; next, the surviving haploid nucleus divides mitotically to form migratory male and stationary female nuclei. With the exchange of the male nuclei and reciprocal fertilization, a new diploid clone is initiated from each member of the pair. The post-zygotic mitotic divisions of the syncaryon produce two macronuclei and two micronuclei; at the first cell division of the new clone, these are segregated so that the two daughter cells are supplied with the normal nuclear apparatus of the vegetative cell, a single macronucleus and a single micronucleus. The macronucleus of the previous sexual generation appears to waste away. Chen has demonstrated reciprocal fertilization in various

ways; his work leads to the conclusion that the two exconjugant clones produced from each pair mating are regularly isogenic.

The fact that at least 97 per cent of all conjugations provide a pair of exconjugant clones expressing identical mating types led Jennings to propose that mating type is genically determined. But Sonneborn[7] and Nanney[8] have suggested that cytoplasmic factors, responsible for mating type determination and reciprocally exchanged during conjugation, might bring about this result. Their argument is weakened, but not invalidated, by the observation that intracellular particles and symbiotic algae are not regularly exchanged during mating.[14]

Mating Type Determination.—The two clones ("synclones") descended as sister exconjugants from a single original pair of conjugating cells are regularly found to express a common mating type (Table 1). Since the synclones are isogenic and massive exchanges of cytoplasm are infrequent, these data provide the first evidence consistent with genic determination of mating type. The rare exceptions to synclonal uniformity are being studied and will be discussed in full in a later publication.

TABLE 1

THE FREQUENCY OF SYNCLONAL UNIFORMITY OF MATING TYPE

	Total Pairs	Pairs Which Yielded Clones Belonging to One Mating Type	Two Mating Types
Jennings' data	587	570	17
Malibu clones	135	133	2
Totals	722	703	19

The four Malibu stocks, mating types A, B, C, and D, were crossed in all six combinations of twos; conjugants were isolated from each mating and a single clone from each pair was raised to sexual maturity and tested for mating type. The results, presented in Table 2, agree with the ratios expected if it is assumed that the genotypes of the stocks are as follows: stock 8, type A, *AaBb;* 25-B, *aaBb;* 32-C, *aabb;* 3-D, *Aabb.*

TABLE 2

THE OBSERVED AND EXPECTED MATING TYPES OF THE DESCENDANTS OF PAIRS FROM INTERSTOCK CROSSES

	Mating Types of F1 Clones								
	Observed				Expected				
Cross	A	B	C	D	A	B	C	D	P
8-A × 25-B	24	21	10	4	22.5	22.5	7.5	7.5	0.5 –0.3
8-A × 32-C	56	76	55	41	57	57	57	57	0.02–0.01
8-A × 3-D	44	11	19	33	40.2	13.4	13.4	40.2	0.3 –0.2
25-B × 32-C	0	40	31	0	0	35	35	0	0.8 –0.7
25-B × 3-D	6	8	14	10	9.5	9.5	9.5	9.5	0.3 –0.2
32-C × 3-D	0	0	45	45	0	0	45	45	1

The cross 8-A (*AaBb*) × 25-B (*aaBb*) should produce mating type B F1 clones with two genotypes, *aaBb* and *aaBB;* similarly, the cross 8-A (*AaBb*) × 3-D (*Aabb*) is expected to yield both homozygous and heterozygous type D progeny (*AAbb, Aabb*). While heterozygous B and D clones crossed to type C should yield 1:1 ratios of the two parental mating types, the homozygotes when crossed to type C would be expected to produce only type B or type D progeny. These predictions are verified by the data presented in Table 3.

Further crosses of the derived homozygous type B and D clones have been carried out. The data in Table 4 are consistent with the hypothesis that F1 clones

8-B, 23-B, 98-D, and 104-D are homozygotes, for they yield only type A among their sexual progeny, and, when crossed to stock 8-A, the parental types, A and B or A and D, appear in a 1:1 ratio.

TABLE 3

MATING TYPES B AND D; HOMOZYGOUS AND HETEROZYGOUS CLONES

Parental Cross	F1 Clone	Progeny Produced in Test Cross, F1 × St. 32-C (aabb) A	B	C	D	Genotype of F1 Clone
8-A × 25-B	6-B	0	9	5	0	*aaBb*
	8-B	0	14	0	0	*aaBB*
	20-B	0	8	8	0	*aaBb*
	23-B	0	17	0	0	*aaBB*
	28-B	0	12	0	0	*aaBB*
	41-B	0	8	7	0	*aaBb*
8-A × 3-D	98-D	0	0	0	18	*AAbb*
	103-D	0	0	8	5	*Aabb*
	104-D	0	0	0	18	*AAbb*
	122-D	0	0	5	8	*Aabb*
	126-D	0	0	0	17	*AAbb*
	129-D	0	0	0	15	*AAbb*
	138-D	0	0	7	9	*Aabb*

TABLE 4

MATING TYPES OF THE DESCENDANTS FROM CROSSES INVOLVING HOMOZYGOUS B AND D CLONES

Cross	Genotypes of Clones Crossed	Progeny Observed A	B	C	D
F1 8-B × F1 104-D	*aaBB* × *AAbb*	13	0	0	0
F1 23-B × F1 98-D	*aaBB* × *AAbb*	23	0	0	0
ST. 8-A × F1 8-B	*AaBb* × *aaBB*	11	19	0	0
ST. 8-A × F1 23-B	*AaBb* × *aaBB*	13	13	0	0
ST. 8-A × F1 98-D	*AaBb* × *AAbb*	34	0	0	16
ST. 8-A × F1 104-D	*AaBb* × *AAbb*	17	0	0	15

As expected, the cross of stock 8-A × F1 8-B (*aaBB*) was found to produce some mating type A clones with the genotype *AaBb* and others with the genotype *AaBB;* the genotypes *AaBb* and *AABb* (both mating type A) were formed in the cross of stock 8-A × F1 98-D (*AAbb*). In each case, these genotypes were identified by crosses to the double recessive, stock 32-C.

Discussion.—The results of the crosses described above serve to establish the following hypothesis: mating type A is determined by the genotypes *AABb*, *AaBB*, and *AaBb;* mating type B is formed by cells genotypically *aaBB* and *aaBb;* the double recessive, *aabb*, is mating type C; finally, the combinations *AAbb* and *Aabb* determine type D. The data provide no evidence for linkage between the two loci. A curious consequence of the breeding system is that the genotype *AABB*, which should determine mating type A, cannot be formed since any mating involves a parental cell which is a recessive homozygote for at least one locus. Can these formulae account for the data presented by Jennings? One is immediately impressed by the fact that most of Jennings' crosses produced progeny predicted by the hypothesis and that genotypes can be readily assigned to the majority of his initial clones. But, as Jennings realized, the behaviour of exceptional clones must be satisfactorily explained before the two locus hypothesis is finally established. The following interpretation shows that these cases do not necessarily contradict the hypothesis.

The genotype of the macronucleus is revealed only by the phenotype expressed by a cell; on the other hand, the genotype of the micronucleus is brought to light

by the results of a breeding analysis. The results described above establish that, regularly, the genotypes of the two nuclei are found to agree; the present explanation of aberrant clones assumes that rare exceptions to this rule may occur. As an extension of ideas earlier set forth by Sonneborn and Orias, we suggest that in certain clones the two known loci concerned with mating type determination become transiently unstable in expression or dominance, and that such instability may lead to an hereditary alteration. Although these alterations are faithfully transmitted through numerous cell generations, their frequency and reversibility tend to set them apart from typical gene mutations. Representative exceptional clones will be discussed in the next paragraphs in light of this interpretation.

Jennings' clone 13 underwent self-differentiation to form mating types A and D. *Prima facie* evidence for instability of macronuclear genes is provided by the fact that the macronucleus is known to determine mating type. When these two branches of clone 13 were allowed to cross, the progeny consisted of 32 A's, 11 B's, no C's, and 17 D's. If the parental A and D subclones are both assigned micronuclei with the genotype *AaBb*, then the observed results are in good agreement with the expected 9:3:1:3 ratio; but if the D subclone is provisionally assigned the micronuclear genotype *Aabb* or *AAbb* (in agreement with its expressed or macronuclear "genotype") then the observed results do not agree with the expected ratio. Hence, this clone provides evidence for instability of expression for genes in the macronucleus.

Aberrant results from other clones analysed by Jennings are brought into line with the two locus hypothesis if instances of variable or unstable allelic expression are assumed. For example, clones 32 and 33, both originally type B, produced subclones of types D and A respectively. Crosses involving these self-differentiated D and A lines reveal that their micronuclear genotypes are *AAbb* and *AaBb*. Unfortunately, the parental type B clones were not bred further but at least the expression of macronuclear genes was altered in these clones.

Several apparent alterations in macronuclear expression have been discovered in crosses of the Malibu strains. As in Jennings' work, sublines from a given clone were found to consist of cells expressing complementary mating types. It does not seem fruitful to comment further on these and other variants so far uncovered since they are few in number and are, at present, incompletely analysed. The point to be stressed is that the appearance of exceptional clones in our material affords the opportunity to analyse in a more satisfactory way the hypothesis of hereditary alteration in gene expression.

A comparison of mating type mechanisms in the more fully studied ciliates suggests a series of variations about a central theme. The simplest and perhaps most primitive (in the evolutionary sense) situation is to be found in *Euplotes patella*, where the genotype of each clone is directly revealed by the expressed mating type and by appropriate crosses. *P. bursaria* and *T. pyriformis* (variety 8) regularly conform to this pattern, and hence genic mechanisms for mating type control have been uncovered by breeding studies; the evidence suggests further that rare variations in the expression of alleles concerned with mating type determination occur. But in other ciliates, these instances of instability are not rare, instead they have become the rule. Consequently, genetic loci concerned with the development of specific mating types cannot be defined; Sonneborn[2, 9] and Nanney[8]

postulate "mutational" changes responsible for the macronuclear differentiation of complementary mating types in *P. aurelia* and *T. pyriformis* (variety 1). Here the genotype of the micronucleus would (if it could!) permit the expression of two or more mating types, whereas the macronucleus in each cell becomes, early in its development, restricted to the expression of but a single specificity. Finally, in *P. multimicronucleatum*, unstable macronuclear expression continues throughout the history of the clone.[15]

The differential expression of alleles determining antigenic specificity has been discovered by Sonneborn *et al.*[16]; in that case, the observed phenotypic variation is brought to light only in aged clones. The dominance of serotype genes in heterozygotes of *T. pyriformis* is apparently indeterminate.[17] The question remains as to whether the basis for these hereditary transitions in gene function lies in an alteration of the primary genetic information (DNA) or only in the degree of expression of an unchanged genetic code. The difficulties in distinguishing genetic from "epigenetic" alterations have been discussed recently by others.[18]

Summary.—Multiple mating types have long been known in *Paramecium bursaria*. It has now been established that a system of complementary genes at two unlinked loci controls each of the four mating types in variety 1. Thus specific genotypes can be assigned to most, but not all, clones. The exceptions do not necessarily invalidate the two-locus hypothesis for they may represent instances of altered genic expression or dominance. If so, the mechanism of mating type determination in *P. bursaria* requires the operation of both "genetic" and "epigenetic" control systems. An evolutionary sequence originating in the genetic control of mating type, as in *Euplotes patella*, through the condition exemplified by *P. bursaria* and *Tetrahymena pyriformis* (variety 8), to epigenetic control, as in *P. aurelia*, *P. multimicronucleatum*, and *T. pyriformis* (variety 1), may be suggested.

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*THE EVOLUTIONARY IMPLICATIONS OF THE CYTOLOGICAL POLYMORPHISM AND PHYLOGENY OF THE VIRILIS GROUP OF DROSOPHILA**

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In 1938 Professor J. T. Patterson embarked on a very intensive collection of Drosophila species because preliminary investigations had convinced him that this fauna was very rich, comparatively unknown, and most suited for the study of the evolution of a genus. The investigations of these species fully justified his conviction (Patterson and Stone[1]). Two of the species groups he and his colleagues investigated extensively were the virilis and the repleta groups of the subgenus Drosophila. Both of these groups have added and continue to add greatly to our understanding of evolution. Flies such as Drosophila leave no fossil record, except for a few recognizable specimens in amber. Mutations are repetitive so the presence of the same allele in different species is hard to prove or to evaluate. However, Professor T. S. Painter's[2] demonstration of the characteristics of the chromosomes of the salivary gland cells of Drosophila and their unique usefulness in analyses of chromosomal abnormalities gave us a tool to analyze the direction and degree of cytological change. With this tool the chromosome phylogeny and variability can be determined and compared to the genetic changes within a species group.

Materials and Methods.—A recently described species, *Drosophila ezoana* (Takada and Okada[3]), and new strains of *Drosophila montana* were available for our study. Genetic and cytological analyses were made by crosses within and between strains of a species together with crosses between different species. The details are to be found in Guest's[4] manuscript and will be published in full later. This paper will present the critical features of the cytological evolution and diversity of this species group, and only a few crosses showing the genetic differences will be presented. The cytological differences between species and the cytological polymorphism within strains were determined in the heterozygotes or hybrids or by comparison to the standard maps of *Drosophila virilis* by Hsu[5] and the standard *Drosophila montana* by Moorhead.[6] Strains of all the species of the virilis group were used in the crosses. In some cases two strains of a species were crossed and the F_1 tested to a third species in the hope that viable offspring would be more probable from the heterotic F_1. The new members of the *montana* species were compared to the standard and giant *montana* for size.

Results.—The new strains of the *montana* from Alaska and Canada proved approximately the same size [estimated using length from head through folded wing tip (mean 4.9 mm, ♀)] as standard *montana* (mean 4.8 mm, ♀) and smaller than giant *montana* (mean 5.3 mm, ♀). Moorhead[6] showed that giant *montana* (which might have been designated a new subspecies) was larger than standard *montana* but did not otherwise differ phenotypically from the standard except for the inversion pattern. The new strains from Alaska and Canada do not differ appreciably from standard in size or other phenotypic characteristics but might be classed as a

different subspecies on inversion pattern differences. Table 1 shows tests to the closely related species of the *montana* complex, *Drosophila lacicola*, *D. borealis* and *D. flavomontana*. The Alaskan and Canadian strains were test mated to the other members of the virilis group including *Drosophila littoralis* (the F_1 female hybrids between the latter and the Smithers' strain were fertile). The variations in fertility are similar to those found by Patterson,[7, 8] by Moorhead,[6] and reported from earlier work by Patterson and Stone.[1]

Drosophila ezoana proved quite unusual in that it is by far the most effectively genetically isolated species in the group. Reciprocal crosses were made with all other members of the group (involving over 20,000 pairs of each sex, including over 6,000 with *Drosophila virilis* and 4,000 with *D. littoralis*). Only four offspring, all F_1 from *littoralis* males to *ezoana* females, survived even as far as large larvae. Table 2 shows that insemination also occurred in the reciprocal cross, and that *virilis* females were more frequently inseminated by *ezoana* males than *ezoana* females were by *littoralis* males (in this cross the alien *ezoana* sperm remained motile in the seminal receptacles of the *virilis* females ten days after the flies were placed together).

The cytological variation in the *virilis* species group including these new forms

TABLE 1

CROSSES OF STRAINS OF *Drosophila montana* FROM ALASKA AND CANADA WITH OTHER SPECIES OF THE MONTANA SUBGROUP

	U. T. No. 2501.2a Yukon				U. T. No. 2503.1 Alaska				U. T. No. 2514.1 Smithers			
	No. of Pairs	No. of Offspring ♀	No. of Offspring ♂	Fertility of F_1	No. of Pairs	No. of Offspring ♀	No. of Offspring ♂	Fertility of F_1	No. of Pairs	No. of Offspring ♀	No. of Offspring ♂	Fertility of F_1
borealis ♀	130	25	31	Fertile	120	2	1	Sterile	120	0	0	..
borealis ♂	120	0	0	..	130	0	0	..	120	2	0	Sterile
lacicola ♀	150	2	6	Fertile	120	0	0	..	120	8	8	Fertile
lacicola ♂	190	256	283	Fertile	150	108	94	Fertile	120	230	248	Fertile
flavomontana ♀	120	25	23	Sterile	110	0	0	..	120	40	43	Fertile
flavomontana ♂	120	0	0	..	120	0	0	..	120	0	0	..

All crosses were made in small mass matings of 10 pairs per vial.

proved most informative (Fig. 1 and Table 3). The *montana* complex which evolved from Primitive III, Figure 1, in North America consists of the three subspecies or forms of *Drosophila montana*, standard, giant, and the Alaskan and Canadian forms, plus three species descended from standard *montana*: *D. lacicola*, *D. flavomontana*, and *D. borealis*. Unfortunately, many of the changes in gene sequence in the X-chromosome in descendants of Primitive III have not been analyzed. We do not know the basic sequences of genes in the X-chromosomes of *ezoana*, *littoralis*, *montana*, or *lacicola*. It would seem probable that five or more inversions were present in hybrids between *ezoana* and *littoralis*, *littoralis* and *montana*, *montana* and *lacicola*, and *montana* and *virilis*. Therefore the total number of inversions analyzed, 92, plus the unanalyzed inversions in the X chromosome, would mean a total of perhaps 120 known in the evolution of members of this species group. The inversions in *Drosophila novamexicana* and the two subspecies *D. americana americana* and *D. americana texana* were worked out especially by Hsu,[5] who also studied *littoralis*, basic *montana*, *lacicola*, *flavomontana*, and *borealis*, and proposed the three primitive types, modified slightly in Figure 1, to account for the cytological phylogeny of the group. The cytological analysis of the giant *montana* strains was

made by Moorhead.[6] Since no rearrangements were found in chromosome 6, it will not be referred to further.

The chromosome phylogeny including the postulated primitive types is shown in Figure 1 and Table 3. In these illustrations we have indicated the inversions at their origin (in a primitive type, a species, or a subspecies) by capital letters. Lower case letters indicate that the inversion occurred in an ancestral form, while letters in italics indicate that the inversion is not fixed but is heterozygous in some individuals. For sample, a particular inversion, B, in the 2 chromosome would be written 2*B*, 2B, 2b, or 2*b*. We have used overlapping inversions where they occur to give direction to the phylogeny as did Sturtevant and Dobzhansky,[9] Dobzhansky and Sturtevant,[10] and Dobzhansky,[11] who reviewed the extensive work done by him and his colleagues. These are not sufficient to establish all relations and we have used the presence of added new inversions, heterozygous and especially homozygous, to establish the direction of cytological evolution. Hsu[5] and Patterson and Stone[1] have presented convincing evidence that *D. virilis* (or Primitive I) was ancestral to the group. The present paper places *D. ezoana* as an intermediate between Primitive III and *D. littoralis*, very close cytologically to the former. The arrows on Figure 1 indicate the direction or more probable

TABLE 2

INSEMINATION OF FEMALES IN CROSSES OF *Drosophila ezoana* WITH OTHER SPECIES OF THE VIRILIS GROUP

♀ ♂	Number of Females Mated*	Number of Females Dissected	Number Inseminated	Number with Motile Sperm
ezoana × *virilis*	500	100	0	0
virilis × *ezoana*	500	100	29	14
ezoana × *littoralis*	260	50	2	0
littoralis × *ezoana*	260	50	2	0
ezoana × *montana*	260	50	0	0
montana × *ezoana*	140	50	0	0

* Crosses made in small mass matings of about 20 pairs each.

direction of cytological evolution. There is no reason to think that we have found all the inversions in any species—for example, the few strains of *D. montana* from Alaska and Canada had 7 new inversions heterozygous. We know 43 inversions which are fixed in one or more species, 41 which originated in a species or the Primitive form which gave rise to it, and 2 which were only heterozygous at their origin but were fixed in a descendant form (2f and 4h). There are additional inversions fixed in X_E, X_{Li}, X_M, and X_{Lc}. Because the complexities of these inversions have so far defied analysis, we can only estimate that there are perhaps 20 to 30 additional inversions fixed in these X chromosomes. Furthermore, there are 51 new inversions heterozygous in the species in which they first occurred. as well as 8 inversions carried over from an ancestral form but still heterozygous.

All of these 92 (+ unanalyzed X inversions) are paracentric except the one pericentric in chromosome 2 which occurred in Primitive III and is present in all descendants of that form. In addition there are present in these species three centric fusions (see Patterson and Stone[1] for summary of analysis): the 3–4 fusion of *D. littoralis*, the 2–3 fusion of *D. americana americana* and *D. americana texana*, and the X-4 fusion of *americana* not found in *texana* except by gene interflow in their overlap zone. No other translocations and only a few minor shifts in

TABLE 3

CHROMOSOME VARIABILITY AND PHYLOGENY OF THE VIRILIS GROUP

Species	Chromosomes and Elements: X / A	2 / E	3 / D	4 / B	5 / C	Inversions: Homozygous New/Old	Inversions: Heterozygous New/Old
virilis	..	..	..	..	..	0	0
Primitive I	..	A	..	..	..	1/0	0
Primitive II	A B	a	..	..	..	2/1	0
novamexicana	a	a	A	A	B	6/3	0
	b	B					
	C	C					
texana	a	a	..	*a*	*A*	0/3	1/4
	b			*b*	*b*		
	c						
americana	a	a	*a*	*a*	*a*	0/3	3/6
	b	*b*		*B*	*b*		
	c	*c*		*C*			
	D						
Primitive III	..	a	..	D	..	0/3	4/6
		D		E		5/1	1/0
		E		F			
		F					
ezoana	X_E	a	..	d	I	1/6	0/1
		d		e			
		e		f		(+X_E)	
		f					
littoralis	X_{Li}	a	H	d	H	5/7	2/0
		d	*I*	e	i		
		e		f	J	(+X_{Li})	
		I		M			
				N			
montana—standard	X_M	a d e f	B	d e f	C	8/7 (2f)	9/0
		G H J K	C	*H G I*	D		
		L	*D*	*L O*		(+X_M)	
				U W			
montana—giant	X_M	a d e f	b c	d e f	c d	0/7 (+8)	14/0
		g h j k	*d L*	*h g i*	*O*		
	J K L	*S T*		*l R S*		(+X_M)	
				T u			
				V w			
				X Y Z			
montana—Alaska-Canada	X_M	a d e f	b c	d e f *h*	c d	0/7 (+8)	7/0
		g h j k	*M*	*g i l*	*o P*		
	k	*U V*		*u w*		(+X_M)	
	N	*W X*		*x z*			
						8/7	30/0
lacicola	X_{Lc}	a d e f g h j k	b c	d e f *h*	c d	4/15	8/1 (4*h*)
		O P Q R	J K	P Q	*K L M N*	(+X_{Lc})	
flavomontana	X_M	a d e f g h j k	b c	d e f h	c d	6/16 (4h)	2/0
		N	E	J *K*	E	(+X_M)	
	E F G						
borealis	X_M	a d e f g h j k	b c	d e f h	c d	3/16 (4h)	4/0
		M	*F G*		F *G*		
	H I						
Homozygous	8 + X_E, X_M, X_{Li}, X_{Lc}	11	7	9	8	41 + X_E, X_M, X_{Li}, X_{Lc} new inversions + 2f and 4h old inversions	
Heterozygous	5	13	6	17	8	49 + 2*f*, 4*H* new inversions + 8 old inversions	
Total	13 + X_E, X_M, X_{Li}, X_{Lc}	24	13	26	16	92 + X_E, X_M, X_{Li}, X_{Lc}	

All forms are compared to *Drosophila virilis*, the cytological standard form. Each chromosome has a series of inversions which are given letters, following the system of Hsu.[5] New inversions are indicated by capital letters; inversions brought in from an ancestral form by lower case letters; and inversions that are not fixed in a species but occur heterozygous in part of the individuals by italicized letters. The accumulation and correlation of inversions show relations and give direction to the phylogeny.

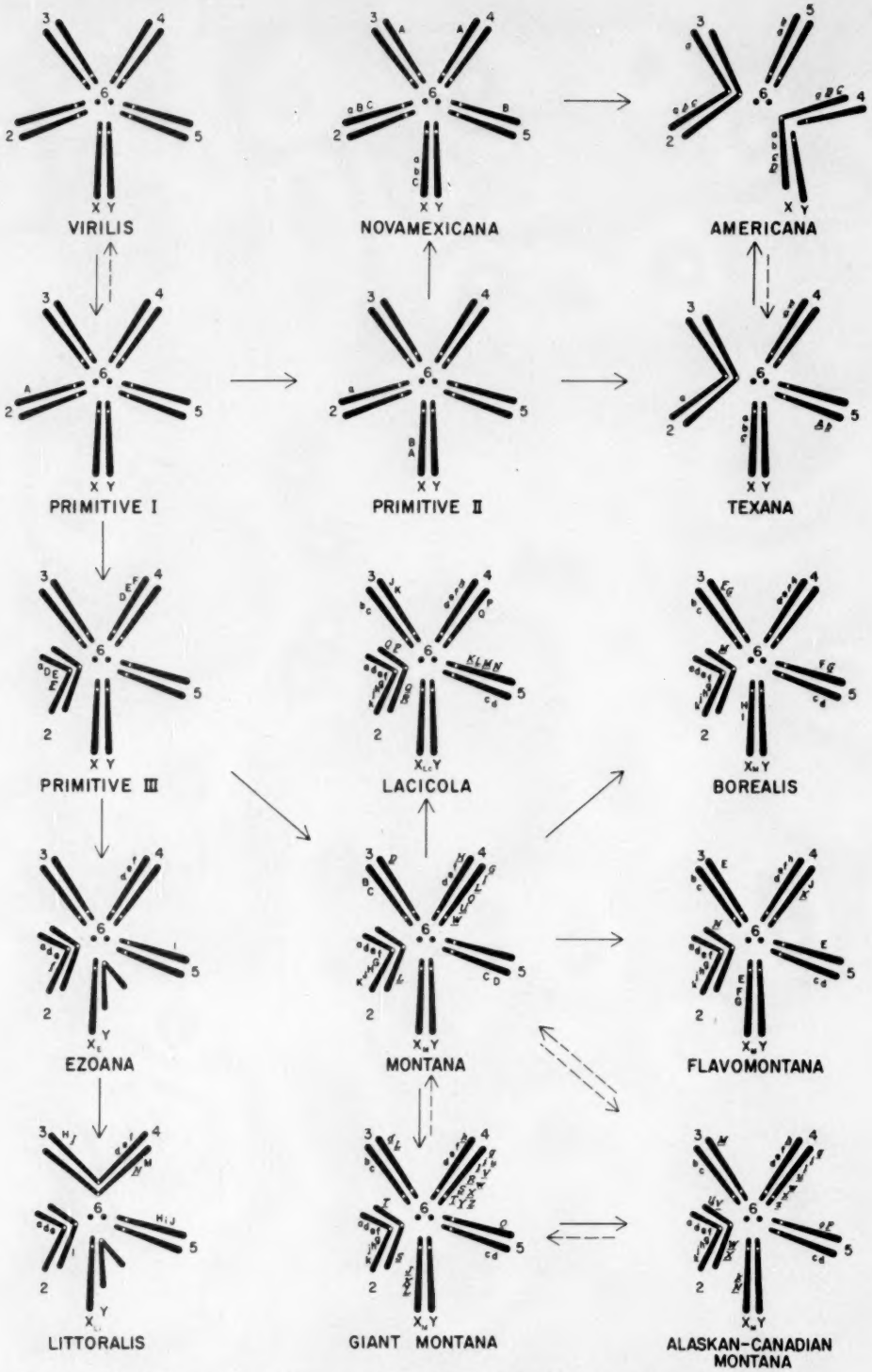

FIG. 1. The chromosome phylogeny and variability of the virilis species group.
Each inversion in a chromosome is given a separate letter. Capital letters mark the origin of an inversion in a species, subspecies, or primitive form; lower case letters indicate the inversion originated in an ancestral form; letters in italics (underscored in the figure), whether capital or lower case, show that the inversion is heterozygous, at least in some individuals. The arrows indicate the more probable evolutionary phylogeny, while arrows each way indicate gene flow between subspecies.

heterochromatin are known. It is interesting to note that the X-4 fusion without a Y and 4 fusion gives *americana* a multiple sex chromosome complex. Nevertheless this is fixed in *americana* and absent from the subspecies *texana* except for gene exchange along the band of overlap of the two subspecies, while no one of the many new paracentric inversions is fixed in one subspecies and absent in the other—Xc of *americana* approaches it most closely. Another interesting observation is that 2*F* occurred only heterozygous in Primitive III (this is the only modification we found necessary in the primitive types as first postulated by Hsu[5]), remains heterozygous in *D. ezoana*, was lost in the evolution of *D. littoralis*, but was fixed in the other line of descent of Primitive III in standard *montana* and its descendants.

Discussion.—Sturtevant and Dobzhansky[9, 10] first used a chromosome phylogeny to establish the sequence relationships in the 3 chromosome of *Drosophila pseudoobscura* and *D. persimilis*. They also indicated that the 3 chromosome of *D. miranda* was related to the other sequences through their hypothetical sequence which differs from the Standard gene sequence by one inversion. Many other inversion differences are present between these three species. Dobzhansky[12] has compared the chromosomes of *Drosophila willistoni* and three of its sibling species and Carson[13] has compared three other members of this species group. Stone[14] has reviewed the studies of chromosome polymorphism in the genus Drosophila and listed most of the extensive investigations up to that time. Despite these many investigations only two real phylogenies that represent major analyses of the chromosome evolution of large species groups exist—the virilis group as discussed here and earlier by Hsu[5] and Patterson and Stone,[1] and the repleta group investigated especially by Wasserman[15] and Wasserman and Wilson.[16] The reason for the success in these two groups and relative lack of success elsewhere is not hard to determine. The inversion changes often involve small inversions, the number of inversion differences between closely related forms is small, nearly the full series of intermediate forms is still in existence, and finally these are quite good species groups for laboratory studies including cytological analysis. For example, comparing *pseudoobscura* and *persimilis* with *miranda* is like attempting to compare *novamexicana* and *texana* with giant *montana* without the known intervening forms. By far the most remarkable chromosome phylogeny is that of the repleta group. Wasserman and Wilson[16] reported the analysis of 22 species of this group. More recently Wasserman[17] has extended the analysis so that he has the cytological phylogeny of 39 species of the more than 60 in the group. Furthermore, he has been able to show the cytological evolution of 7 other species not at present included in the group from the common cytological stem gene sequence. The ancestor(s) of the repleta group achieved some basic adaptation to desert conditions which led to a tremendous multiplication of species in the sparsely populated Southwest and especially Mexico. In these species genetic evolution was rapid, cytological evolution was not. For example, Wasserman has now recognized 100 inversions in chromosome 2, which is homologous to element E and hence to chromosome 2 of the virilis group; in the latter, consisting of nine species plus three subspecies or separate varieties, there are 24 inversions in this chromosome. The general problem of chromosome homologies in Drosophila was developed by Muller,[18] and Sturtevant and Novitski.[19] Inversion phylogenies have a unique advantage in that they

show the steps, but not the number of species in the steps, in the evolution of a group even if members and their steps are missing.

The chromosome variability in the genus has been reviewed by Stone[14] so only a few added comparisons with the data on the virilis group will be given. Most of the inversion variability in *pseudoobscura* is in chromosome 3, element C. This element is not especially variable in the virilis group. There are 50 known inversions found heterozygous in *willistoni* alone and one individual was heterozygous for 16 of them, (da Cunha, Dobzhansky, Pavlovsky, and Spassky[20]). These authors point out that there are now known the following heterozygous inversions in this species, given by chromosome arm and element: XL(A) = 11, XR(D) = 7, 2L(B) = 8, 2R(C) = 6, 3(E) = 18. The most variable species of the virilis group, *montana*, consisting of three varieties or subspecies, has the following series of heterozygous inversions, given by chromosomes and elements to facilitate comparison: X(A) = 4, 4(B) = 14, 5(C) = 2, 3(D) = 3, 2(E) = 7, total = 30. The three sibling species of *montana* are also quite variable, as are the sibling species of *willistoni*. The largest number of heterozygous inversions present in one strain (perhaps involving more than one pair as parents) of giant *montana* was 8 (Moorhead[6]) in contrast to 16 in one individual in *willistoni*. Da Cunha *et al.*[20] repeat their deduction that chromosomal polymorphism of paracentric inversions exists because of selective advantage. With this conclusion we must agree. The simplest general observation supporting it is the paucity of pericentric inversions and translocations (even the relatively innocuous centric fusions) which like paracentric inversions are two-break rearrangements and should therefore occur in as great numbers. However, the infrequent and absent types are known to have varying degrees of disadvantage when heterozygous (and they would originate heterozygous) because most of them produce aneuploid gametes at meiosis (Patterson and Stone[1]). Even paracentric inversions involving the heterochromatin which so often gives rise to position effect mutations that are often detrimental are very rare in Drosophila. The theoretical basis was developed by Fisher[21] over twenty years ago, who pointed out that slightly less than 2 per cent of unique mutational events would survive even with 1 per cent selective advantage, while no mutation without selective advantage would survive indefinitely from a single occurrence.

Da Cunha *et al.*[20] state: "The question which unavoidably presents itself, is, what causes some populations to be so much more polymorphic than others? Da Cunha, Burla, and Dobzhansky (1950)[22] advanced the working hypothesis that the chromosomal polymorphism is adaptive, and the populations which exploit a greater variety of ecological niches in the territory which they inhabit are more polymorphic than populations restricted to a narrow range of ecological opportunities."

It might be well to state the relations somewhat differently. Regions with many varied ecological niches have these filled in time by living systems, sometimes by several species and sometimes by one with great adaptive capacities which may depend on genetic polymorphism, whereas regions with serious ecological restrictions must impose similar restrictions on the variability of the genotypes. Furthermore, genotypes from a marginal restricted habitat might be able to invade an area with rich and varied ecological niches much more effectively than a genotype from a rich area could invade an area of much more restricted and intense selection, despite

population pressure which causes major migrations from densely populated centers to peripheral regions.

Carson[23] (and earlier publications) has suggested that marginal populations are homozygous because homozygosity allows maximum recombination. This is undoubtedly true but certain additional facts must be considered. For example, the restriction of crossingover in some chromosome regions by heterozygous inversions has repeatedly been demonstrated to increase recombination in others, Schultz and Redfield.[24] Furthermore, the mere effect of difference in population size must have an effect. In a very large central population the number of individuals with any chromosome or large segment of a chromosome homozygous must be very much larger than in the peripheral populations. Therefore, the number of recombinations will be much greater in the central population (although not as great a per cent), than in peripheral areas. In fact, if these new combinations are especially beneficial and dominant in their effect, they may be multiplied as inversion heterozygotes protected from immediate breakdown by crossingover until they can build up in frequency. J. F. Crow (personal communication) pointed out a second and more important reason for questioning the greater importance to survival of homozygosity of peripheral populations. The importance of a sexual system and of recombination over an asexual system increases with population number. Further consideration stresses the importance of the role of selection against the ill-adapted migrants from the population centers in establishing the genotypes of the peripheral populations. The evidence that necessarily peripheral populations connected to parent populations give rise to new forms and species does not seem sufficient. As Carson pointed out, *Drosophila robusta* does not have such budded-off species. Rather it seems that isolation plus restricted population size are both necessary. This has been discussed and the genetic effects elaborated and illustrated in bird evolution by Mayr[25] and for insects and invertebrates in general by White.[26] Both of these seem to be necessary factors in the evolution and elaboration of the repleta species group in Drosophila in addition to the desert adaptation of the ancestral form. Probably most of the more than 60 species in the repleta group evolved in the desert and semidesert conditions of Mexico and the Southwest, an environment where both isolation and initial restriction in size would occur frequently.

There are some interesting conclusions to be drawn from comparisons of the cytological evolution and polymorphism of different species groups with very different population distributions and densities. Wharton[27] made the first extensive analysis of the role of fusions, pericentric inversions and changes in heterochromatin in the genus. Further adequate discussions are to be found in Patterson and Stone[1] and Stone.[14] We shall deal with the role of paracentric inversions, both fixed and heterozygous, in the evolution of the species group. Wasserman[15–17] has investigated the repleta group, particularly in the southwest United States, Mexico, and Central and South America and the Antilles around the Caribbean Sea. These are usually small populations, particularly in the desert areas, where most of them occur. The 39 analyzed members of the group are characterized by relatively few inversion differences between species. Even so, there are decidedly more inversion differences fixed between species than occur as heterozygotes within species. The virilis group includes species with very small populations (e.g.,

novamexicana), those which are small, and *virilis* which in Asia seems to have large populations. This last species and the most restricted species, *novamexicana*, seem to be cytologically homozygous, whereas the others are variable. Spieth[28] demonstrated that *lacicola* and *borealis*, which are sympatric in northern Minnesota, live in a restricted high humidity belt around lakes. Other members of the group found in North America are also restricted to banks of streams and lakes. Along desert streams of the southwest the collections of *novamexicana* have been few so we must defer final decision—so far it is homozygous for gene arrangements although the three populations investigated for heterotic properties by Stone, Alexander, and Clayton[29] prove to have some very interesting genetic differences.

Drosophila pseudoobscura and *persimilis* have more inversions heterozygous in their populations than the number of fixed inversion differences. However, the restricted species with comparatively small populations, *Drosophila miranda*, differs from these two by many more fixed inversions than the total of variable inversions in the three species. Unfortunately for an analysis, the species of the willistoni group are too diverse cytologically to determine the number of inversion differences fixed between them. The species *willistoni* has one of the larger if not the largest population distribution in the genus, perhaps being the largest species in numbers and large population distribution of any amenable to genetic analysis.

Perhaps the most significant finding is that there is no direct correlation between number of individuals in a species and number of heterozygous (unfixed) inversions in a species. *Drosophila pseudoobscura*, with a widespread distribution and large populations in western North America, has somewhat fewer inversions heterozygous than *montana*, although the latter species is at present characterized by small linear populations spread over a considerable area. A guess, which certainly overestimates the relative number of *montana*, would estimate their ratios as *montana: pseudoobscura: willistoni* = $1:10^4:10^8$. Nevertheless there are known only 50 heterozygous inversions in the latter species but 30 in *montana*, even though *willistoni* has been sampled much more extensively. Perhaps this is good evidence that the difficulties discussed by Haldane,[30, 31] in terms of the conditions for coadaptation in polymorphism for inversions together with the cost of natural selection, limit the number of cumulatively heterotic gene combinations associated with inversions that may be expected in a population. Dobzhansky and Pavlovsky[32] claim that the inversions in certain populations of *pseudaobscura* are heterotic because of genes with *specific combining ability*, and that different populations differ in this respect so that the same inversions are not necessarily successfully interchangeable between populations. This can hardly be the case in the tremendous populations of *willistoni* with a number of inversions heterozygous in each chromosome for da Cunha *et al.*[20] point out that individuals with the same set of inversions are rare. In *willistoni* inversions must be retained by heterotic genes which have *general* and *cumulative combining ability*. This seems to be the case also in *montana*. Several inversions may be heterozygous in a strain but we do not find consistent heterozygotes between certain gene sequences in a population, so far as our more limited sampling allows us to determine.

The effective uniqueness of each inversion and its loss if it lacks selective advantage (Fisher[21]) allows us to make some interesting calculations about the number of inversions which has been utilized in the evolution of the genus and also

the number which occurred but was lost. Stone[14] estimated the number of inversions fixed in the evolution of the genus using the number fixed per species in the virilis and repleta groups compared to the number heterozygous in those and other groups. The number of variable (heterozygous) inversions then numbered 592 in the 42 species which had been studied. The virilis and repleta groups differ in ratio of fixed to heterozygous inversions from 1.2:1 for the former to 2:1 for the latter, as now determined by Wasserman.[17] The number of species in the genus was earlier estimated at 650 but M. R. Wheeler now estimates that there are two to three times that number. On the basis of the 650 species, the number of inversions fixed in the evolution of the genus was estimated at between 6,100 and 36,500. This was a very conservative estimate as no additional allowance was made for greater cytological differences between than within species groups or subgenera. With the larger number of species now proposed for the genus, the number of paracentric inversion differences fixed in its evolution is probably between 22,000 to 56,000, while perhaps 18,000 to 28,000 are now heterozygous in the many populations around the world. If we assume that the selective advantage of the inversions in their initial struggle to be established was one per cent, this would mean that from 1.1 to 2.8 million inversions with this much selective advantage occurred, but the remainder were eliminated. This leaves the vast majority of inversions, conservatively ten or one hundred times as many, which did not have selective advantage, or were disadvantageous, that also were eliminated. When we remember that inversions are relatively unique events whereas mutations occur and recur time and again, we get some further idea of the tremendous numbers of trials of rearrangements and mutations in different combinations and sequences which have gone into the evolution of even this small genus. These repeated and varied trials with natural selection and time enough to absorb the cost (Haldane[31]) make evolution inevitable, as Fisher[21] pointed out. Wright[33] has reviewed his concepts of the necessity of a balanced array of forces (mutation, selection, population structures, etc.) and of the importance of complex balanced genotypes and their shifts to higher selective peaks. We have presented some evidence on the results of variations in population structure and the tremendous array of variability available through time to make possible the changes necessary to reach more highly adapted genotypes.

Summary.—The virilis group of the genus Drosophila has been revised and extended. Strains of *Drosophila ezoana* from Hokkaido and new strains of *Drosophila montana* from Alaska and Canada have been tested and compared to earlier tests with members of the group. *D. ezoana* is isolated genetically more than any other member of the group. Cytologically it is very close to the postulated Primitive III in gene arrangement and in part validates this postulated ancestral form. The strains of *montana* from Canada extend the known range and cytological variability of this species. The several strains proved to be genetically diverse as had other strains of *montana*. The virilis group consists of nine species. The chromosome phylogeny has been worked out so that we know the lines of evolution and the necessary primitive forms (cytologically) to fill out the phylogeny, Figure 1. *Drosophila virilis* has been demonstrated to be both genetically and cytologically nearest the primitive ancestor of the group. In addition to the normal *virilis* arrangement of genes in the X and the four major autosomes, there are 92 known and

analyzed heterozygous or homozygous inversions in this group (plus 20 to 30 more which are fixed, i.e., homozygous, in the X chromosomes of *Drosophila ezoana, montana, lacicola, and littoralis*). There are 43 analyzed plus these added X chromosome inversions which have been fixed in the evolution of one or another member of this species group. There are now known 49 inversions which are present sometimes in some species but not fixed (plus 2f and 4h which are variable in some species but fixed in others). The amount of cytological variability cannot be predicted from the present population of a species since more unfixed, sometimes heterozygous, inversions occur in *montana* than in *pseudoobscura* but less than in *willistoni*. *Drosophila montana* is much less frequent than the other two species and, like other members of the virilis group, is restricted to the high humidity belt along streams or lakes.

There exist now two extensive chromosome phylogenies in the genus Drosophila where the direction and extent of cytological variation in the evolution of the species has been thoroughly established. These are the virilis group discussed here, consisting of 9 species plus 3 subspecies or varieties (Figure 1) and in addition, there is the remarkable repleta group and its relatives which has been analyzed, particularly by Wasserman. He will soon publish a revised phylogeny which includes now 39 species in the repleta group plus 7 species in closely related groups. The extensive genetic evolution but very conservative cytological evolution has made this possible.

It is most remarkable that only 100 years after Darwin published *The Origin of Species* we have established so many of the genetic mechanisms involved in evolution. Perhaps it is even more remarkable that we have been able to establish the direction and extent of cytological changes in the well-established cytological phylogeny of 9 species in one species group and 46 species in a complex involving several other species groups.

We wish to thank Dr. Eizi Momma who brought us the strains of *Drosophila ezoana* from Hokkaido; Dr. D. D. Miller and Mr. Chris Dahlie, who collected the strains of *Drosophilia montana* in Canada and Alaska; Dr. Marvin Wasserman, who allowed us to include some of his unpublished data on the repleta group; Dr. M. R. Wheeler, who discussed these general problems and checked the manuscript; and Dr. J. F. Crow, who read the manuscript and made several suggestions. Professor J. T. Patterson began the work on the virilis group with us and continues to contribute inspiration and interest in these problems.

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*SOME HOLOMORPHIC SEMI-GROUPS**

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In this note we state some basic spectral properties of a class of operators associated with a representation of a Lie group. We merely indicate the proofs since we intend to give details in another context.

Let $T(.)$ be a strongly continuous representation of the Lie group $\mathfrak{G}$ in the Banach space $\mathfrak{X}$. For a in $\mathfrak{A}$, the Lie algebra of $\mathfrak{G}$, let $A(a)$ be the infinitesimal generator of the one-parameter group, $T(exp(ta))$, $-\infty < t < \infty$, and let $A^*(a)$ be the adjoint of $A(a)$. It is known that

$$\mathfrak{W}_k = \{x \epsilon \mathfrak{X} \,|\, x \epsilon \text{Domain}(A(a_1) \ldots A(a_k)) \text{ for all } a_1, \ldots, a_k \epsilon \mathfrak{A}\}$$

is dense in X and that

$$\mathfrak{W}_k^* = \{x^* \epsilon \mathfrak{X}^* \,|\, x^* \epsilon \text{ Domain } (A^*(a_1) \ldots A^*(a_k)) \text{ for all } a_1, \ldots, a_k \epsilon \mathfrak{A}\}$$

is dense in $\mathfrak{X}^*$ in the weak* topology. Let $\{e_1, \ldots, e_n\}$ be a basis for $\mathfrak{A}$ and set $A_i =$

$A(e_i)$, $A_i^* = A^*(e_i)$. If $\alpha = (\alpha_1, \ldots, \alpha_k)$, $1 \leq \alpha_t \leq n$, is a sequence of integers we denote a product such as $A_{\alpha_1} \ldots A_{\alpha_k}$ by A_α; $|\alpha| = k$ is the length of α. The operator $B^\circ = \Sigma_{|\alpha| \leq m} a_\alpha A_\alpha$ (the coefficients are complex numbers) is defined on $\mathfrak{W}_m$ and, denoting α in reversed order by α^*, the operator $\tilde{B}^\circ = \Sigma_{|\alpha| \leq m} a_\alpha A^*_{\alpha^*}$ is defined on $\mathfrak{W}_m{}^*$. The closure B of B° and the weak* closure $\tilde{B}$ of $\tilde{B}^\circ$ are well-defined operators. If for any real n-vector, $\xi = (\xi_1, \ldots, \xi_n)$, $|\Sigma_{|\alpha| \leq m} a_\alpha \xi_\alpha| \geq \rho \|\xi\|^m$ ($\rho > 0$ is fixed and $\|\xi\|$ is the ordinary Euclidean norm) then

THEOREM 1. *The adjoint B^* of B is $\tilde{B}$.*

To prove this we observe that if $B^* x_1^* = x_2^*$ then,[1] for x in $\mathfrak{X}$, $x_1^*(T(g)x)$ is a weak solution of the equation

$$\Sigma_{|\alpha| \leq m} a_\alpha R_{\alpha^*}\{x_1^*(T(g)x)\} = x_2^*(T(g)x);$$

R_i is the infinitesimal transformation

$$R_i f(g) = \lim_{t \to 0} t^{-1} \{f(\exp(te_i)g) - f(g)\}.$$

We use the properties of weak solutions of elliptic equations to show that x_1^* is in $\mathfrak{W}_{m-1}{}^*$ and then apply an adaptation[2] of the mollifier technique.

If the stronger condition

$$(-1)^{j-1} \mathrm{Re}\{\Sigma_{|\alpha| \leq m} a_\alpha \xi_\alpha\} \geq \rho \|\xi\|^m,$$

with $2j = m$, is fulfilled then

THEOREM 2. *The operator* B *is the infinitesimal generator of a semi-group,* S(t), *of class $H(\phi_1, \phi_2)$, for some ϕ_1 and ϕ_2.*[3]

The theorem is equivalent to an inequality $\|R(\lambda, B)\| \leq M/[\rho(\lambda, S)]$ for the norm of the resolvent of B; M is a constant and $\rho(\lambda, S)$ is the distance of λ from a sector

$$S = \{\zeta \,|\, \psi_2 \leq \arg(\zeta - \zeta_0) \leq \psi_1\}, \quad \frac{\pi}{2} < \psi_2 \leq \pi \leq \psi_1 < 3\pi/2.$$

To obtain the inequality we observe that

$$x^*(T(g)(B - \lambda)x) = \Sigma_{|\alpha| \leq m} a_\alpha L_\alpha x^*(T(g)x) - \lambda x^*(T(g)x),$$

with

$$L_i f(g) = \lim_{t \to 0} t^{-1}\{f(g \exp(te_i)) - f(g)\};$$

partially invert this equation in a neighborhood of the identity using a parametrix; and then establish appropriate estimates[4] for the parametrix. It is of interest to note that if a_α is real when $|\alpha| = m$, $S(t)$ is holomorphic in a half-plane.

The semi-groups, $S(t)$, have a canonical representation. Let μ be a left-invariant Haar measure on $\mathfrak{G}$, then

THEOREM 3. *There is a unique function* h(t, g) (*defined for* t *in an open sector containing* $(0, \infty)$) *in* $L_1(\mu)$ *for each* t *and such that for all representations* T(.) *of* $\mathfrak{G}$

$$S(t)x = \int_{\mathfrak{G}} h(t, g) T(g) x \mu(dg).$$

The integral is a Bochner integral and $h(t, g)$ is analytic in t and g. We remark finally that, when t is in the interior of the domain of $S(.)$ and x is in X, $S(t)x$ is an analytic vector.

The above results generalize theorems of Nelson[5] and Nelson and Stinespring.[1]

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*THE EXTENT OF ASYMPTOTIC STABILITY**

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In studying the stability of a system it is never completely satisfactory to know only that an equilibrium state is asymptotically stable or for that matter to know only that it is unstable. In a mathematical sense it may be asymptotically stable but from a practical point of view be unstable, and, conversely, it may be mathematically unstable but practically stable. Both stability and instability are local concepts. An equilibrium state of a system may be unstable, and yet it may be true that the system always tends to return, perhaps not to the equilibrium state itself, but sufficiently near the equilibrium state. An equilibrium state can be asymptotically stable and yet perturbations which would be considered small compared to the perturbations to be expected may cause the system to drift away from the equilibrium never to return. As a practical matter, it is necessary to have some idea of the size of the region of asymptotic stability. It is never possible to do this by examining only the linear approximation to the system. The effect of nonlinearities must be taken into account, and Liapunov's stability method[1] provides a means of doing this. The purpose of this paper is to report some mathematical theorems that underlie methods for estimating regions of asymptotic stability. These methods, with examples illustrating them, are to be discussed elsewhere in greater detail.[2]

The system whose stability is being investigated is described by the vector differential equation

$$\dot{x} = X(x). \tag{1}$$

The state of the system at time t is an n-vector $x(t) = (x_1(t),\ldots,x_n(t))$. The phase velocity $dx/dt = \dot{x}$ is defined by the vector field $X(x) = (X_1(x),\ldots,X_n(x))$. For each initial state x° we assume there is a unique solution $x(t)$ of (1) satisfying $x(0) = x^\circ$, and that this solution depends continuously on the initial state x°. The equilibrium state being investigated is at the origin: $X(0) = 0$. The Liapunov method depends upon the construction of a suitable domain Ω and a suitable

Liapunov function $V(x)$, which is a kind of generalized energy function. We assume throughout that $V(x)$ has continuous first partials in Ω. With reference to the system (1), we define

$$\dot{V}(x) = \frac{\partial V}{\partial x_1} X_1 + \ldots + \frac{\partial V}{\partial x_n} X_n = (\text{grad } V) \cdot X.$$

If $x(t)$ is a solution of (1), then

$$\frac{d}{dt} V(x(t)) = \dot{V}(x(t)).$$

No knowledge of the solutions of (1) is required to compute $\dot{V}(x)$. It is computed directly from a knowledge of the differential equations which describe how the system changes. The difficulty lies in constructing $V(x)$. This requires experience and technique—a technique in which Russian mathematicians and engineers excel. The theorems stated below describe what are suitable Liapunov functions for determining the extent of asymptotic stability. The basic result is

THEOREM 1. *Let Ω be a bounded closed set with the property that every solution of (1) starting in Ω remains for all future time in Ω. Suppose there is a scalar function $V(x)$ with the property that $\dot{V}(x) \leqq 0$ in Ω. Let E be the set of all points in Ω where $\dot{V}(x) = 0$. Let M be the largest invariant set in E. Then every solution starting in Ω approaches M as $t \to \infty$.*

In some instances the construction of a Liapunov function $V(x)$ will itself guarantee the existence of a set Ω. For instance,

THEOREM 2. *Let Ω denote the closed region defined by $V(x) \leqq l$. If, in addition Ω is bounded and $\dot{V}(x) \leqq 0$ in Ω, then every solution starting in Ω approaches M as $t \to \infty$. (M is set defined in Theorem 1.)*

Note with regard to Theorem 2 that if $V(x) \to \infty$ as $||x|| \to \infty$, then the set Ω defined by $V(x) \leqq l$ is bounded for all values of l. If $\liminf_{\|x\| \to \infty} V(x) = l_0$, then Ω is bounded for all $l < l_0$.

Thus, under suitable circumstances, the set Ω is an estimate of the region of asymptotic stability. According to Theorem 1 the procedure is to find a region Ω and a suitable function $V(x)$. If $\dot{V}$ does not vanish identically along any solution starting in Ω except the origin, then every solution in Ω approaches the origin at $t \to \infty$. It seems in some examples to be easier to separate the problems of finding Ω and constructing a Liapunov function $V(x)$ although as Theorem 2 points out the Liapunov function itself may determine Ω.

If the origin is stable and every solution approaches the origin as $t \to \infty$, then the system is said to be *completely stable* (asymptotically stable in the large). The region of asymptotic stability is the whole space. The basic theorem leading to methods for establishing complete stability is

THEOREM 3. *Let $V(x)$ be a scalar function with continuous first partials for all x. Assume that*

i. $V(x) > 0$ *for all* $x \neq 0$
ii. $\dot{V}(x) \leqq 0$ *for all* x.

Let E be the set of all points where $\dot{V}(x) = 0$ and let M be the largest invariant set contained in E. Then every solution bounded for $t > 0$ approaches M as $t \to \infty$.

To establish the complete stability of a system one needs (i) to establish that all solutions are bounded for $t \geqq 0$ and (ii) to construct a function $V(x)$ satisfying the conditions of Theorem 3 and such that M is the origin. Here again one may be able to conclude from the Liapunov function $V(x)$ itself that all solutions are bounded for $t \geqq 0$. This is true, for instance, if $V(x) \to \infty$ as $||x|| \to \infty$, although it often is easier to consider (i) and (ii) as separate problems. Boundedness is a type of stability and can itself be investigated by Liapunov methods.[3]

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*VIRUS-CELL INTERACTION WITH A TUMOR-PRODUCING VIRUS**

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The polyoma(PY) virus or parotid tumor agent[1, 2]—a DNA-containing virus[3, 4]—is characterized by a duality of action: it produces neoplasias of various types in different species of rodents,[5] and causes cell degeneration in mouse embryo tissue cultures.[6] In the experiments to be reported here, it was possible to obtain in cellular cultures *in vitro* the oncogenic effect of the virus; this afforded the possibility of studying the relationship between the oncogenic and cytocidal effect of the virus. The results so far obtained reveal a situation novel in animal viruses and suggest the existence of a host-virus interaction with characteristics reminiscent of temperate bacteriophage.

Material and Methods.—The PY virus was obtained from Dr. Rowe of the National Institutes of Health. A stock was prepared from a single plaque and serial passages of this stock in mouse embryo tissue cultures were used for the experiments. In these passages, the virus maintained both the cytocidal activity in mouse embryo tissue cultures and the property of eliciting heart, liver, and kidney sarcomas within a few weeks after injection into newborn Golden hamsters. The virus was assayed by plaque formation on mouse embryo monolayer cultures by means of the technique previously described.[7]

Cultures from trypsinized whole embryos of either Swiss mice or Golden hamsters were prepared as previously described.[7] All experimental cultures were secondary cultures at the time of the first exposure to the virus. Plaque assays were done on monolayer cultures of mouse embryo cells which had been serially transferred two to five times. The growth medium for the cells consisted of reinforced Eagle's medium[8] supplemented with 20 per cent calf serum. Experimental cultures were grown in Eagle's medium supplemented with 10 per cent calf, horse, or fetal bovine serum, as indicated later.

Results.—The multiplication of the virus: Confluent monolayer cultures of either mouse or hamster cells were infected with virus at a multiplicity of about 10 mouse plaque-forming units per cell. After an adsorption period of forty minutes, the layers were washed to remove most of the non-adsorbed virus, and were covered

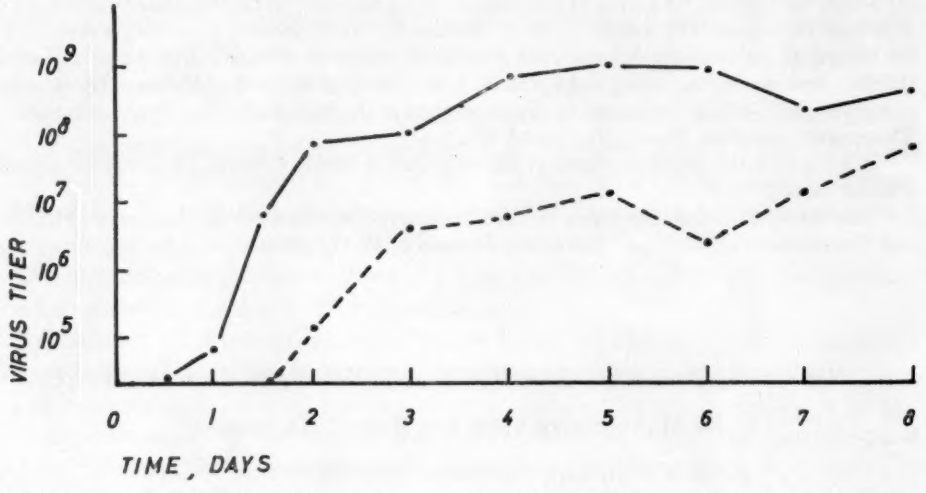

FIG. 1.—Extra-plus intracellular virus per culture at various times after infection.——Mouse embryo culture. – – – Hamster embryo culture. Fluid changes were done on the third and sixth day after infection. The growth medium was Eagle's medium supplemented with 10 per cent horse serum.

with Eagle's medium supplemented with 10 per cent of either calf, horse, or fetal bovine serum. The cultures were subsequently incubated at 37°C and the virus in the supernatants and in the cells (after disruption by three cycles of freeze-thawing) determined at various time intervals.

The growth curves obtained are reported in Figure 1, where the titer of intra-plus extracellular virus per culture is given. The curves show a long latent period, as found for another tumor-producing virus, the Rous virus.[9] The virus production was initially much greater in the mouse cultures; after about a week the difference decreased. At that time, however, the hamster cultures, which had continued to grow, contained many more cells than the mouse cultures, so that the average production of virus per cell was always much smaller in the hamster cultures.

Early behavior of the infected cultures (first week): The mouse cultures appeared unchanged for the first day after infection. Infected cultures containing a number of cells sufficiently low to allow the counting of the cells *in situ* showed after 24 hours a threefold increase in cell number, equal to that of uninfected cultures. The

cell number stopped increasing on the second day after infection. Degenerative changes became visible in the majority of the cells during the third and fourth day, simultaneously with the increase in virus titer. A proportion (about 20%) of the cells, however, remained unaltered as already observed by using fluorescent antibodies.[10] These unaltered cells multiplied in the subsequent days—in fact, frequent mitoses were observed—while more cells underwent degeneration: a balance of these two processes maintained the culture in a steady state.

The hamster cultures, in contrast, continued to increase in cell number during the whole week after infection. Cell counts on parallel cultures with lower cell concentrations showed no difference in the division rate of infected and control cultures for six days of observation. Neither did the infected hamster cultures show obvious signs of degeneration. Since, by plating the cells several hours after infection, most cells proved to be virus-yielders, it must be concluded that they released virus in small quantities without degeneration.

Late behavior of the infected cultures: The mouse cultures remained in the steady state for about four weeks. During this period the virus titer in the supernatants remained constant at a level of about 10^8 PFU per culture, in spite of the fluid changes. This prolonged existence of the steady state in the cultures is by itself of special interest and is being investigated in greater detail. The observations can, at present, be interpreted as follows: It is unlikely that the multiplying cells belonged to a virus-resistant type which was originally present in the culture, since such cells would have replaced the sensitive cells in a few days. It is also unlikely that the multiplying cells represented either partly resistant cells—like those occurring in several virus-carrying cultures of poliovirus[11] and Newcastle disease virus[12]—or a non-infected fraction of sensitive cells. In both cases, in fact, they would have been infected after a few days by the progeny virus accumulating in the medium in large amounts. It is therefore concluded that the surviving cells are infected cells which do not undergo degeneration. The conclusion that the surviving cells are altered cells is also indicated by the finding that all cells of a mouse embryo culture infected with PY virus become resistant to vesicular stomatitis virus.[10] It seems, furthermore, that the cells carry the virus in a state in which the fate of the virus-cell complex is undetermined (see below). Cells containing the virus in this "uncommitted" state can divide but have a considerable chance of being killed by a shift of the virus to the state of extensive, cytocidal multiplication.

A gradual change was observed in the mouse cultures after four weeks. The proportion of degenerating cells decreased significantly together with the titer of intra- and extracellular virus of the cultures. The cell number increased and weekly transfers became possible. The outgrowing cells were of a new type, more elongated than the original cells and with a tendency to form interwoven net-like structures when the cultures became more crowded. Ten weeks after infection, the cultures appeared to be made up entirely of the new cell type. They could be transferred twice weekly, contained no more degenerating cells than normal ones, and produced little virus.

The hamster cultures, at the end of the first week after infection, were made up of a single dense layer of cells indistinguishable from the controls. During the second week a striking difference between the infected and control cultures became apparent. Whereas the number of cells in the control cultures appeared to increase

only slightly during this time, the infected cultures continued to increase in cell number to a value approximately three times that of the control cultures. This led to strong acidification of the medium of the infected cultures. The new cells were more elongated than the control cells and had a tendency to grow in interwoven netlike structures similar to those described above for the infected mouse cells. Whorls of heavy cell strands became noticeable above the continuous cell layer in the infected cultures, but not in the control cultures.

After fourteen days, the cultures were dispersed with trypsin for injection into animals (see below), or for further transfers. No difference between the growth rate of control and infected cultures was observed during the first week after the transfer. In the second week after the transfer, however, whorls of heavy cell strands again appeared in the infected cultures but not in the control cultures; at the same time the medium became strongly acidified. The virus titer decreased drastically in the transformed cultures. Frequently no extra- or intracellular virus could be detected; in other cases titer of 10^3 PFU or less per 10^6 cells were obtained.

This proliferative response in hamster embryo cells to PY virus was first observed in experiments carried out in collaboration with Dr. G. Freeman. It has since been observed and followed in four independent experimental series, occurring in calf or fetal bovine serum medium but not in horse serum medium. The proliferative response failed to appear in two other experimental series. The observations tend to show that the occurrence of a proliferative response depends on the physiological state of the cells at the moment of infection, a point which is under further investigation.

Nature of the transformed cells: As to the nature of the new cells which became established both in infected mouse and hamster cultures, it is unlikely that they were originally present; in fact, no selective conditions favoring multiplication of such cells were present in the hamster cultures in which no cell destruction took place. It is more likely that these new cells were cells of the original type transformed or converted by the virus. Since the transformed cells of the mouse cultures became established in the presence of high virus concentrations, it can furthermore be assumed that these cells are resistant to superinfection with PY virus. This conclusion is also supported by the fact that superinfection of transformed hamster cells with PY virus failed to give evidence of virus production during the first week after infection.

The ability of the transformed cells to grow under conditions unfavorable to the growth of the control cells suggested that they were of neoplastic nature. To test this possibility, 10^6 to 4×10^6 cells of six independently transformed hamster embryo cultures were inoculated under the skin of 18–20 day old hamsters. All six cultures gave rise to tumors at the site of inoculation; five tumors were palpable six days, one tumor two weeks, after the inoculation. All six tumors grew progressively without any signs of regression. In three cases, the animals were killed when the neoplasias had reached a diameter of about 3 cm. The neoplasias were well localized; no metastases or tumors at other locations were found. Histologically, the neoplasias were constituted by fusiform cells. Fragments of the tumors were trypsinized, and tissue cultures were prepared from the cell suspensions. The outgrowing tumor cells formed whorls similar to those of the original cultures whenever the cell layers became crowded. Frequently no virus or only very small

amounts (100 PFU per 10^6 cells) of virus could be demonstrated in these cultures.

As controls, more than sixty hamsters of the same age were inoculated with 10^6 to 10^7 cells of uninfected hamster embryo cultures from the same culture batches in which the transformed cultures had been contained. In none of the hamsters were nodules formed. Four infected cultures in which no *in vitro* transformation had been observed likewise failed to induce tumor formation in the animal. Neither did ten hamsters inoculated with 5×10^7 to 10^8 PFU of PY virus form any tumors at the site of inoculation. The hamsters are still alive and it is therefore unknown whether they formed tumors of the organs normally affected by PY virus.

Conclusions.—In these experiments, the PY virus gave rise to two types of virus-cell interaction: a cytocidal interaction, leading to extensive virus synthesis and cell degeneration, and a moderate interaction leading to the transformation of the cells into neoplastic cells, usually unable to produce detectable virus and resistant to superinfection with the same virus. The cytocidal interaction is most frequent in the mouse cultures, the moderate interaction in the hamster cultures. The different reactions of the mouse and hamster cultures reproduce and clarify the events occurring in the animal: it is in fact known that in the newborn mouse the virus produces extensive cell degeneration and only later—after several months—tumors; whereas in the newborn hamster the virus produces few degenerative phenomena but within a few weeks leads to formation of tumors. The results obtained extend those of Dawe and Law[13] in showing that differences in virus-cell interaction explain the various effects of the virus in the animal, including the disappearance of the virus from hamster tumors.[14, 15, 16]

The following hypothesis is put forward to explain the results of our experiments: upon entering a cell, the virus assumes an "uncommitted" state in both hamster and mouse cells, during which it may undergo a limited multiplication without appreciably affecting the normal properties of the cell. From this "uncommitted" state, the virus has the choice of entering either the state of cytocidal multiplication or the integrated state. The late transformation of the mouse cultures would be due to the selection of a few transformed cells. In the hamster cultures, on the other hand, the choice would be almost exclusively toward the integrated state, both in the animal and in the tissue culture.

The state of the virus in the transformed cells is unknown. Two properties of cultures of these cells, i.e., the absence or low level of virus production and the resistance to superinfection, are similar to the properties of lysogenic bacterial cultures and suggest that the integrated virus exists as provirus. Other hypotheses are, however, not excluded: the virus could ultimately be lost from the transformed cells and resistance to superinfection could be a secondary consequence of the transformation.

The transformed, neoplastic cells are able to grow above the monolayer to which the normal cells are usually confined. This suggests that *in vitro* the normal cells are still subjected to growth-regulating mechanisms from which the transformed cells can escape. This property of PY-transformed cells, which is also characteristic of cells transformed by the Rous virus, may be general for neoplastic cells *in vitro;* if so, it may open a wider approach to the study of neoplasia-producing viruses.

These experiments will be reported in detail elsewhere.

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THE STABILITY OF NON-DISSIPATIVE COUETTE FLOW IN THE PRESENCE OF AN AXIAL MAGNETIC FIELD

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1. The effect of an axial magnetic field on the stability characteristics of Couette flow in the limiting case of zero viscosity and infinite conductivity has been examined recently by Chandrasekhar.[1] He showed that an adverse gradient of angular velocity can always be stabilized by a sufficiently strong magnetic field and that the required field strength can be derived from the solutions of the related nonmagnetic problem. Within the framework of the "small gap" approximation, the nonmagnetic problem has recently been solved exactly,[2] and in this paper, therefore, we present a determination of the magnetic field strength required to completely stabilize the flow.

2. In the small gap approximation it is assumed that the gap, $d = R_2 - R_1$, is small compared to the mean radius, $R_0 = {}^1/_2 (R_2 + R_1)$. The angular velocity distribution can then be approximated by the linear profile

$$\Omega = \Omega_1 [1 - (1 - \mu)\zeta], \tag{1}$$

where

$$\mu = \Omega_2/\Omega_1 \quad \text{and} \quad \zeta = (r - R_1)/d. \tag{2}$$

In the absence of a magnetic field, Rayleigh's criterion shows that the angular velocity distribution (1) is unstable for $\mu < 1$ and we wish to determine, therefore, the magnetic field strength required to stabilize the flow under these conditions.

3. By considering an axisymmetric perturbation of the velocity distribution (1) whose t- and z-dependence is of the form

$$\exp [i(pt + kz)], \tag{3}$$

we find that the linearized equation for v, the radial component of the perturbed velocity, can be reduced to the form

$$\left(p^2 - \frac{H^2}{4\pi\rho} k^2\right)(D^2 - a^2)\, v = 2\,\Omega_1^2\,(1 - \mu)\,\frac{R_0}{d}\,a^2[1 - (1 - \mu)\zeta]v, \tag{4}$$

where

$$a = kd \qquad \text{and} \qquad D = d/d\zeta. \tag{5}$$

In equation (4), the magnetic permeability has been set equal to 1 to avoid confusion with the present use of μ to denote the ratio of angular velocities of the cylinders. The boundary conditions are

$$v = 0 \text{ at } \zeta = 0 \text{ and } 1. \tag{6}$$

For the related nonmagnetic problem, we have the equation

$$(D^2 - a^2)\, v = -a^2\lambda[1 - (1 - \mu)\zeta]v \tag{7}$$

with

$$\lambda = -2\,\Omega_1^2(1 - \mu)\,R_0/d. \tag{8}$$

Thus, if the characteristic values $\lambda(a;\ \mu)$ of equation (7) are known, then the solution of equation (4) can be written in the form

$$p^2 = k^2\left[\frac{H^2}{4\pi\rho} - \frac{2\Omega^2(1 - \mu)R_0 d}{a^2\lambda(a;\ \mu)}\right]. \tag{9}$$

From the solution of equation (7), which can be expressed quite easily in terms of Airy functions, it is found that for all positive characteristic values of λ, $a^2\lambda$ is a monotonic increasing function of a. Accordingly, if we let

$$\Lambda(\mu) = (1 - \mu)\lim_{a\to 0}\frac{1}{a^2\lambda(a;\ \mu)}, \tag{10}$$

then the minimum field strength, H_{min}, required to stabilize the flow is given by

$$\frac{H_{min}^2}{4\pi\rho} = 2\,\Omega_1^2 R_0\, d\,\Lambda(\mu). \tag{11}$$

4. For small values of $1 - \mu$, $\Lambda(\mu)$ has the linear behavior

$$\frac{\Lambda(\mu)}{\frac{1}{2}(1 + \mu)} = \frac{1 - \mu}{n^2\pi^2}\,[1 + O(\epsilon^2)], \tag{12}$$

where

$$\epsilon = \frac{1 - \mu}{\frac{1}{2}(1 + \mu)} \tag{13}$$

and $n = 1, 2, \ldots$ denotes the mode. For large values of $1 - \mu$, it has the asymptotic behavior

$$\Lambda(\mu) \sim \frac{1}{(-x_n)^3\,(1-\mu)}, \tag{14}$$

where x_n are the zeros of the Airy function $\mathrm{Ai}(x)$ in Miller's notation[3] and $n = 1, 2, \ldots$ again denotes the mode. For values of $1 - \mu$ intermediate between these two extremes, one must employ the detailed solution of equation (6).

5. For the lowest mode of instability ($n = 1$), an evaluation of $\Lambda(\mu)$ from the exact solution of equation (7) leads to the results shown in Figure 1. These results would not appear to be unreasonable when we recall that for $1 - \mu < 0$ the flow

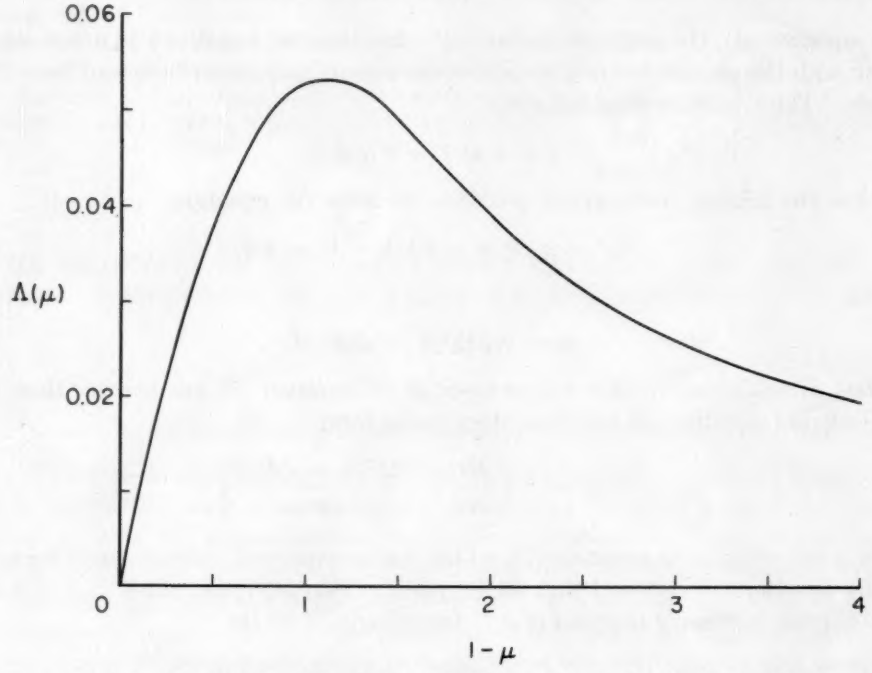

FIG. 1.—The behavior of $\Lambda(\mu) = (H_{\min.}^2/4\pi\rho)/(2\Omega_1^2 R_0 d)$ for the first mode of instability ($n = 1$).

is already stable in the absence of a magnetic field and that for large values of $1 - \mu$ the region of instability is confined to a narrow region adjacent to the inner cylinder. Thus, for both small and large values of $1 - \mu$, the magnetic field strength required to stabilize the flow becomes vanishingly small.

From Figure 1 it is seen that there exists a critical value of the magnetic field strength which is just sufficient to stabilize the flow for arbitrary rates of rotation. This critical value of the field strength is

$$\frac{H_{cr}^2}{4\pi\rho} = 0.1063\ \Omega_1^2\ R_0\ d \tag{15}$$

and occurs for

$$1 - \mu_{cr} = +\,1.1275. \tag{16}$$

The ratio of $H^2/4\pi\rho$ to $\Omega_1^2 R_0 d$ is a measure of the relative importance of magnetic and inertia forces. The result (14) shows, therefore, that if this ratio exceeds the critical value of 0.1063, then the flow will always be stable.

I am indebted to Professor S. Chandrasekhar for some helpful comments. This work was supported by the Office of Naval Research.

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*EXCESSIVE GROWTH OF THE SYMPATHETIC GANGLIA EVOKED BY A PROTEIN ISOLATED FROM MOUSE SALIVARY GLANDS**

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Communicated by Viktor Hamburger, January 4, 1960

The discovery that mouse sarcomas produce a diffusible agent which selectively promotes the growth of the sensory and sympathetic ganglia in the chick embryo,[1, 2] confronted us with three major problems: (*a*) the characterization of the growth agent, (*b*) the analysis of its mode of action on the embryonic nerve cells which were increased in number and in size under the impact of the agent to an extent unmatched by any previous experimental device, and (*c*) the question of the significance and distribution of nerve-growth agents in tumors and other tissues.

These problems were investigated using the method of explanting embryonic ganglia *in vitro* together with the tumor or other tissues to be tested. The finding that fragments of tumor explanted in proximity to the ganglia elicited exuberant nerve fiber outgrowth, and that this effect is apparent within 5–6 hours, greatly speeded up and simplified the bio-assaying procedures, thus making possible the biochemical approach to our problem.[3] The isolation of a nerve-growth promoting protein fraction from the neoplastic tissue was the first step in this direction.[4] In experiments aimed at the further characterization of the nature of the agent, Cohen made use of snake venom as a source of proteolytic enzymes. This led to the discovery that the snake venom also contains a nerve-growth promoting factor which replicates in all respects the effects of mouse sarcomas on the sensory and sympathetic ganglia of the chick embryo *in vitro* and *in vivo*.[5, 6] Since the snake venom is produced in a modified salivary gland, the mouse salivary glands were tested as another possible source of the nerve-growth promoting factor. The extract of the mouse submaxillary glands proved to contain a nerve-growth promoting agent even more potent than the two mentioned above.[7, 8] It was found that this agent likewise promotes the growth of the sensory and sympathetic embryonic ganglia of the chick

embryo, leaving other systems unaffected. The similarity extends also to other facets of the phenomenon such as the massive neurotization of the viscera of the chick embryo. The potency of this factor, its ready availability, its presence in various tissues of different vertebrates, suggested an investigation of its effects on a wider scale. In all previous experiments the chick embryo was the test object. We have now investigated the effects of the nerve-growth factor in mammals. The results of *in vitro* experiments on mouse and rat sensory and sympathetic ganglia were reported.[7, 8] We will report in this paper the results of *in vitro* effects on human fetal ganglia and of *in vivo* experiments on the sympathetic ganglia of newborn, young, and adult mice. We will also report on experiments designed to establish the relationship between salivary glands and nerve-growth agents. In a second paper, we will present evidence of the near-total disintegration of the sympathetic ganglia in mammals as a result of daily injections of the antiserum to the nerve-growth factor. The agents used in all these experiments were prepared by Dr. S. Cohen and they are described by him in another paper in this issue.

In vitro Effects on Sensory and Sympathetic Ganglia of Human Fetuses.—Four human fetuses, three about two-and-a-half months old and one about three-and-a-half months old, were obtained from the Medical School of Washington University. They resulted from hysterotomy for therapeutic abortion and were made available as soon as delivered.† The sensory and sympathetic ganglia of these fetuses were explanted in tissue culture: the same hanging drop technique was used as in all previous experiments.[3, 5, 7] The medium consisted of one part of chicken plasma, one part of synthetic medium, and one part of the purified salivary fraction. Control cultures were prepared using one drop of physiological solution instead of the salivary fraction. A total of forty experimental and forty control cultures were prepared; in each culture a large number of ganglia were explanted. The effects of the salivary extract on ganglia of the two-and-a-half month fetuses were similar in all respects to the effects elicited by mouse sarcomas, snake venom, and the mouse salivary extract on ganglia of chick embryos (Fig. 3), mouse, and rat embryos. A dense halo of nerve fibers surrounded the ganglia in the experimental cultures; it was already apparent 12 hours after the beginning of the experiment and increased in density and in size at the end of the first day (Fig. 5). A comparable, but somewhat slower, reaction was observed in ganglia explanted from the older fetus. A considerable degree of liquefaction of the cultural medium took place on the second and third day of culture; the experiments were then discontinued. Control ganglia showed few or no nerve fibers in the area surrounding the explant 24 hours after the preparation of the culture (Fig. 2).

In vivo Effects on the Sympathetic Ganglia of Newborn and Adult Mice.—(*a*)*Material and methods:* The effect of the salivary extract was tested on newborn and adult mice. Solutions of the active extract at different concentrations and different degree of purification were at first assayed in three groups of newborn mice and then on adult mice.

Group 1: Ten newborn mice were injected daily with the salivary extract referred to in Cohen's paper as fraction CM-1 (these PROCEEDINGS, this issue). This solution was injected subcutaneously in the amount of 0.05 ml per gm of body weight at a concentration of 1,500 units of biological activity per ml. One unit of biological activity is defined as that amount per ml required to elicit a 3+ response

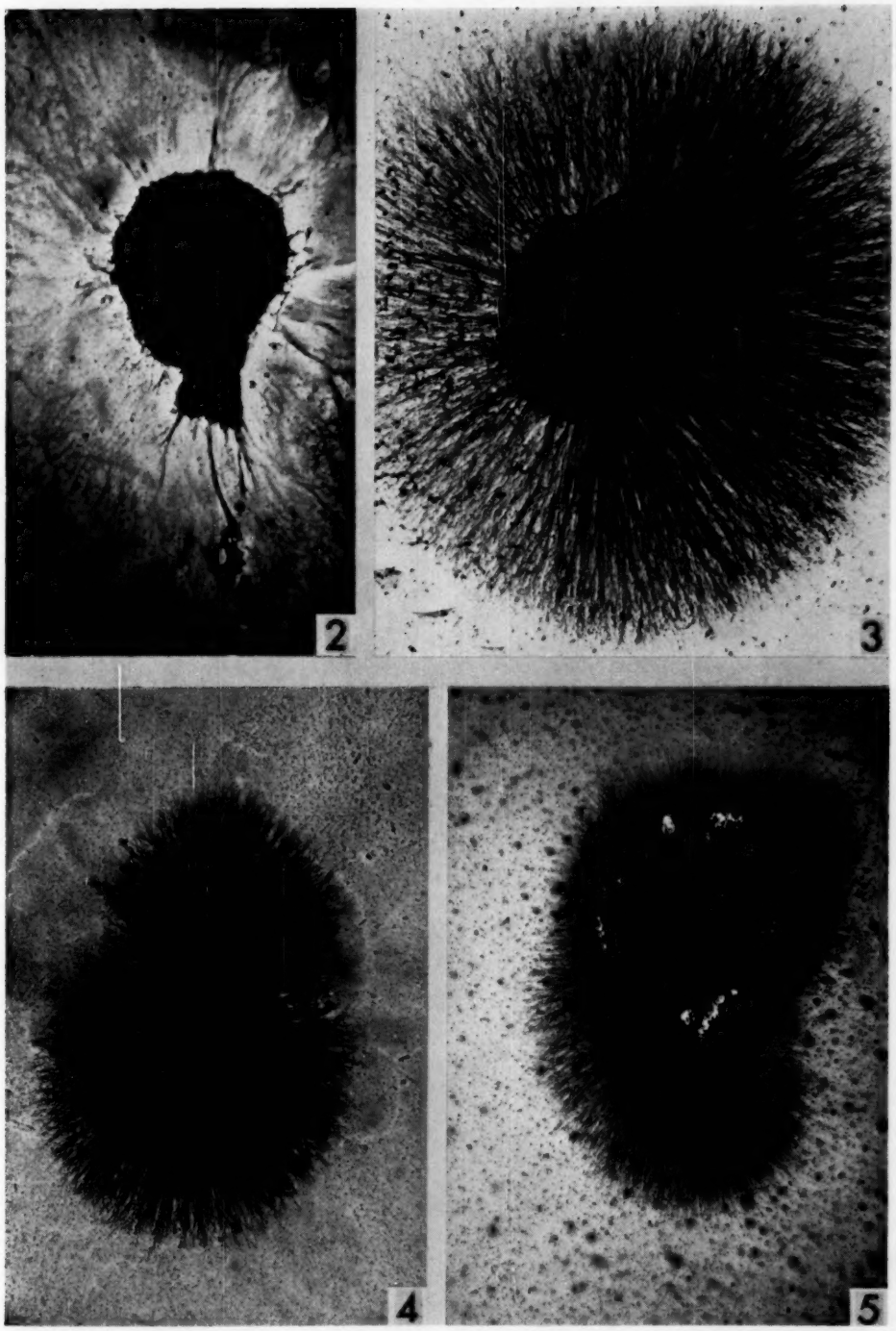

PLATE I.—Microphotographs of sensory ganglia after 24 hours *in vitro*. Silver impregnation. FIG. 2.—Ganglion of a $2^1/_2$ months human fetus in the standard control medium. FIGS. 3, 5.—Ganglia of a 7-day chick embryo (Fig. 3) and of a $2^1/_2$-month human fetus (Fig. 5) in a medium containing the purified salivary protein at a concentration of 1:18,000. FIG. 4.—Ganglion of a 7-day chick embryo in a medium containing mouse serum at a concentration of 1:30. Short and dense halo in Figs. 4 and 5 characteristic of strong and slightly inhibitory effects of the growth agent at high concentration.

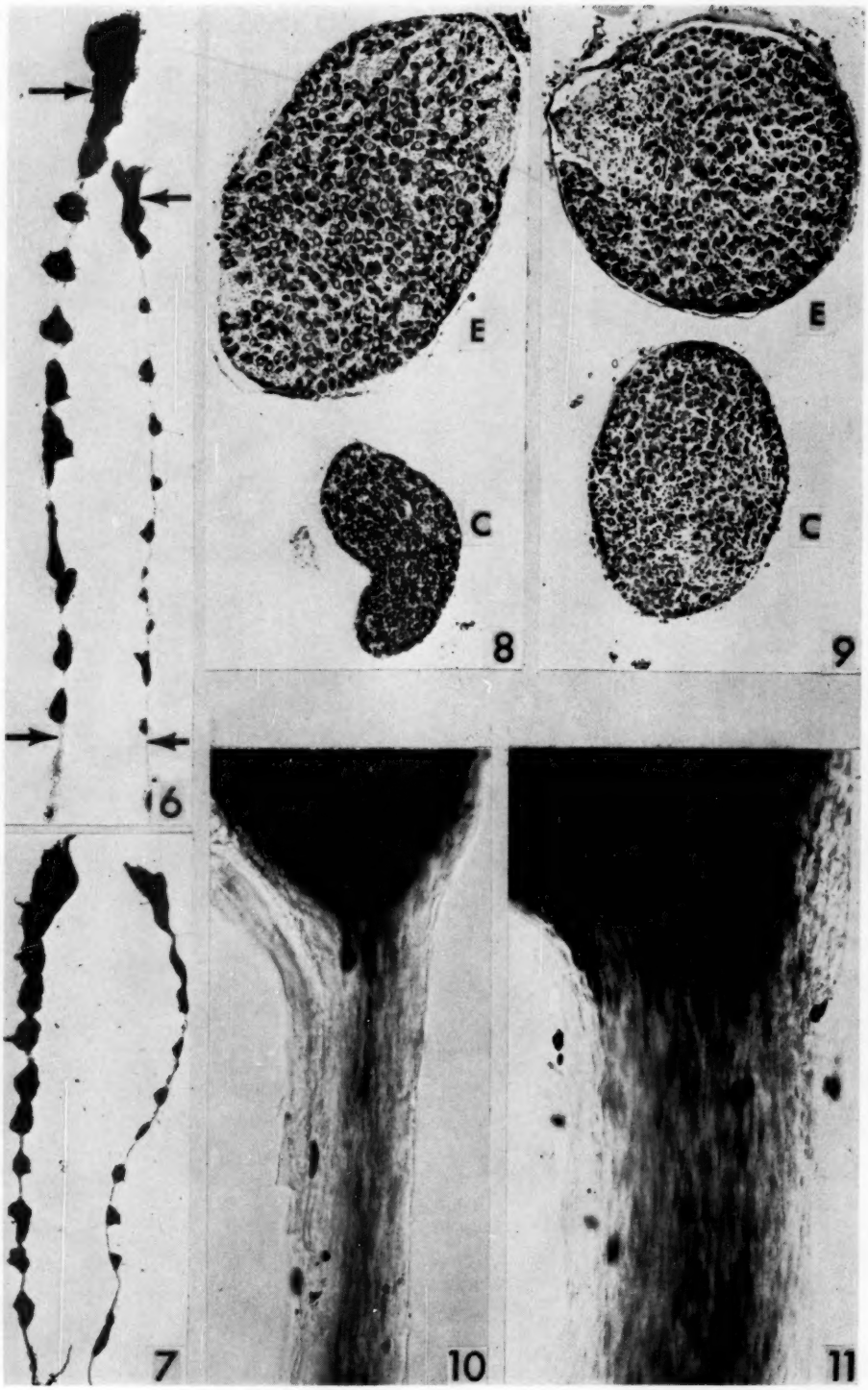

PLATE II.

FIGS 6, 7.—Whole mounts of the sympathetic thoracic chain ganglia of experimental (E) and control (C) mice 19 and 12-day old respectively. Experimental mice injected with the CM-3 salivary fraction since birth. FIG. 8.—Transverse section of stellate ganglia in experimental (E) and control (C) ganglia of Fig. 6. Sections through levels indicated by upper arrows in both chains of Fig. 6. FIG. 9.—Transverse section of superior cervical ganglia of two adult mice. E, mouse injected for 1 week with the CM-1 salivary fraction. C, control ganglion. FIGS. 10, 11.—Sympathetic nerve trunk of control and experimental chain ganglia of Fig. 6e at higher magnification. Low arrows in Fig. 6 indicate areas enlaged in Figs 10, 11.

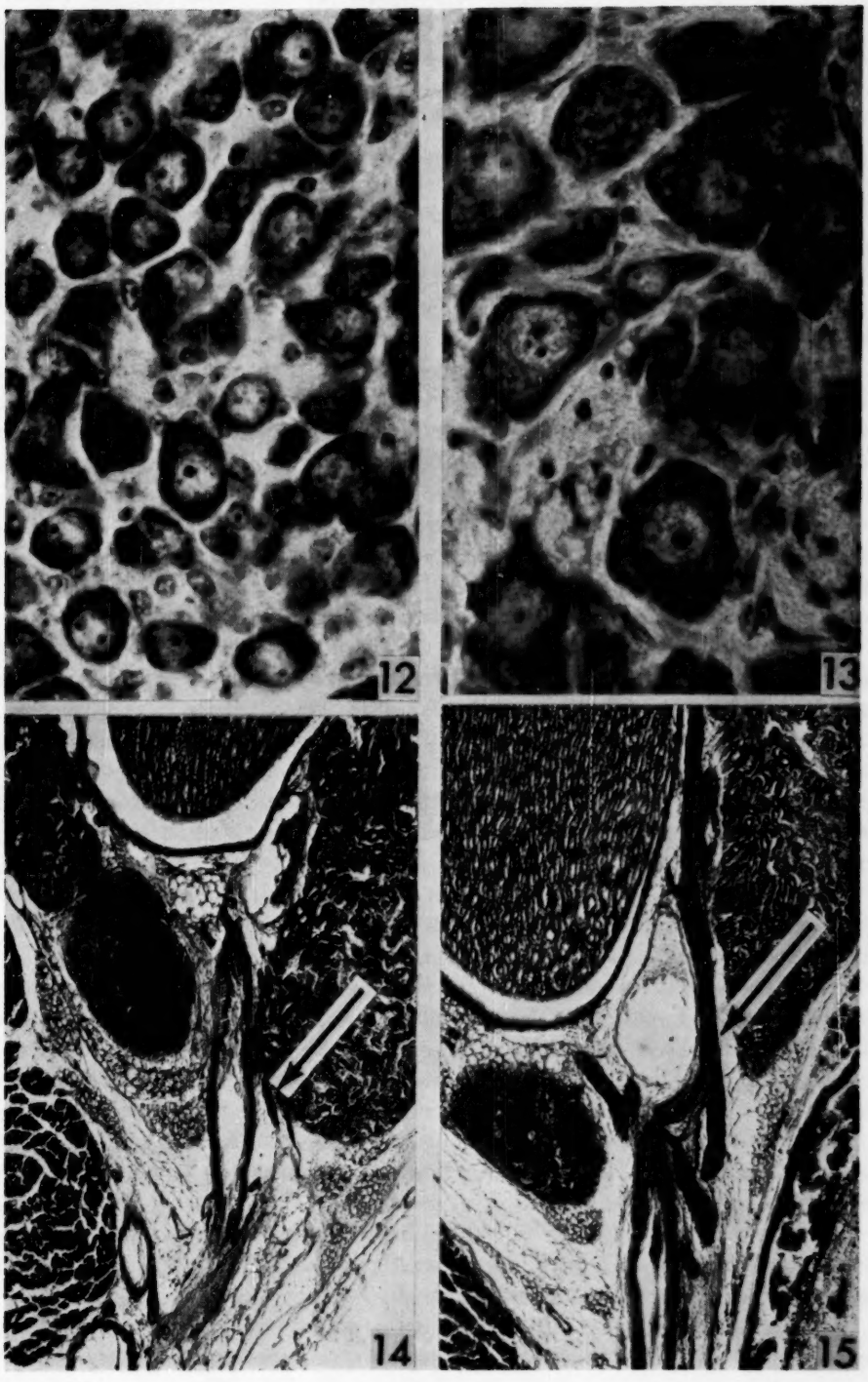

PLATE III.

FIGS. 12, 13.—Comparison of cell size in control and experimental stellate ganglia represented in Figs. 6, 8. Toluidin blue stain. FIGS. 14, 15.—Comparison of size of sympathetic nerve along the renal artery in control (Fig. 14) and experimental 19-day old mouse (Fig. 15) injected daily with the CM-3 salivary fraction since birth Arrows point to nerve in both figures. Cajal De Castro silver impregnation.

in tissue culture. The injected and control mice of the same litters were sacrificed between the 7th and the 14th day and examined for effects on the nervous system and other systems. When it was found that such treatment had consistently elicited a significant increase in the sympathetic ganglia, a second series of experiments was performed with a more concentrated solution.

Group 2: Thirty newborn mice were injected with the CM-1 fraction at a concentration of 6,000 units per ml. The solution was injected daily, in the same amount as used in previous experiments. The injected and untreated mice of the same litters were sacrificed every day or every other day between the second and the thirtieth day.

Group 3: Ten newborn mice were injected daily, from birth, with a more highly purified fraction referred to as CM-3 fraction in the paper by S. Cohen. The solution was injected in the same amount and in the same concentration as in the experiments of Group 2. The injected and control mice were sacrificed between 1 and 9 days and at 12 and 19 days respectively.

Group 4: Fifty adult mice were injected with the CM-1 fraction at the same concentration and in proportional doses per body weight as in groups 2 and 3. The injected and control mice were sacrificed in groups of 3 to 4 between the end of the first week and the fourth week and compared with controls.

The injected and control mice of all 4 groups were dissected and used for investigation of the sympathetic ganglia; other components of the nervous system were also examined. The sympathetic ganglia were either dissected out and stained with hematoxylin and toluidin blue for counts of mitotic figures and of nerve cells, or they were left *in situ* and studied after silver impregnation and sectioning of the whole organism. This material was used for the study of the neurofibrillar differentiation of nerve cells and of the peripheral distribution of sympathetic nerve fibers in injected and control animals.

Counts of mitotic figures were made in sympathetic ganglia of mice of the third group between 12 hours and 9 days. The mitoses were counted in each section of the superior or stellate ganglia in control and treated mice.

Area measurements were made in ganglia dissected from mice 5 to 27 day old, of groups 2 and 3. Each section was projected with the help of the camera lucida and the contour of the section was drawn on cardboard. The total number of sections was then weighed and the weight compared with that of the same control ganglion. Since the weight is proportional to the volume, the figures indicate a similar ratio between the volume of experimental and control ganglia. A total of 18 control and 18 experimental ganglia were measured and compared. The same technique was used on a small number of ganglia of the fourth group.

Cell counts were performed in sympathetic ganglia of groups 3 and 4, by inserting a micrometer disk in the ocular and counting all nerve cells in every other section of the experimental and control ganglia.

Two additional series of experiments were performed. In the first series we tested the serum of adult and weanling mice for the presence of the nerve-growth factor. A total of 150 adult mice of both sexes and of 30 weanling mice were tested. The blood was either collected from the blood vessels immediately after decapitation of mice in light chloroform anesthesia, or it was drained directly from the aorta and the heart in mice anesthetized with nembutal. The blood was

allowed to clot at room temperature and then stored for half an hour in the refrigerator. The serum was then collected in separated vials and each specimen tested on sensory ganglia of 8-day chick embryos explanted *in vitro* with the usual hanging drop technique. All sera were also tested at dilutions of 0.1, 0.01, and 0.001.

In the second series of experiments we extirpated the submaxillary and sublingual salivary glands in 25 adult and 5 weanling mice, and we inspected the sympathetic ganglia of the operated and control mice between 2 weeks and 6 months after the operations.‡

(*b*) *Effects of the purified salivary extract on newborn mice:* The newborn mice injected with the CM-1 fraction in the weak and even more in the strong concentration, exhibited side effects which will be mentioned but not described in detail. From the third day on, the growth rate dropped sharply and at the end of the first month, the mice were barely larger than at the end of the first week. The hair growth was severely impaired, the lids opened 6 to 7 days earlier than in controls and the cutting of the inferior and superior incisors and their calcification took place 5 days earlier than in controls. The animals recovered if the injections were discontinued at the end of the first month; dwarf mice resumed growth and three months later they did not differ from untreated controls at a macroscopical inspection. The effects mentioned above did not affect the vitality of the mice in the doses used. None of the forth injected mice died as a result of the treatment. No side effects were observed in newborn mice injected with the more purified fraction CM-3. They were healthy and vigorous as controls. Obviously, we are dealing with 2 factors, one of which was removed in the process of purification.

Both fractions CM-1 and CM-3 evoked a marked overgrowth of the sympathetic ganglia (Figs. 6, 7, 8, 9). In the following we will consider only the results of the injections of fractions CM-1 and CM-3 in the concentration of 6,000 units per ml (groups 2 and 3).

The average *volume increase* of the superior cervical ganglia in group 2, as determined by comparison of 13 experimental and 13 control ganglia was 3 : 1. The corresponding figures for the same ganglia of mice injected with fraction CM-3 and controls (group 3) were higher: measurements of 3 experimental and 3 control ganglia at 12 days gave a ratio of 4.1 : 1 and on 2 experimental and 2 control ganglia at 19 days gave 6.4 : 1. More long range experiments are planned to establish the ceiling of this effect.

The results of *cell counts* in 12 control and 12 experimental ganglia of mice injected since birth with the CM-3 fraction are given in Table 1. The increase in cell number in the injected mice over the controls averages 2.5 and 2.18 respectively in one 12- and one 19-day old mouse. The results of cell counts in adult mice, as given in the same Table for 5 controls and 2 experimental ganglia, show no increase in cell number in the injected adult mice over the controls. It is of interest to note that the total cell population in the normal stellate ganglia is 13,000 whereas the same ganglion in an experimental mouse has a population of over 30,000. The mechanism of this cell increase will be dealt with below.

The concomitant *increase in cell size* in injected animals is shown in Figures 8 and 9 for the stellate ganglia of the 19-day old mice and adult mice respectively. No size measurements were made. Since the volume increase in the ganglia was 4 to 6

times (see above) whereas the increase in cell number in the same ganglia was about twofold, we conclude that cellular hypertrophy has a greater share in the end effect than the increase in cell number.

Cytological examination showed that the hypertrophic neurons differ from controls also in the more intense basophilia and in the size of the nucleoi which are much larger than in controls (Figs. 12, 13). A parallel increase in neurofibrillar material in the hypertrophic nerve cells is apparent in the silver-stained ganglia. Observations of ganglia dissected from three-day old mice injected with the CM-1 or CM-3 fractions since birth, indicate that the size increase is already evident at that time.

It was of interest to decide whether the increase in cell number in the injected mice is due to an increase in *mitotic activity* or to other mechanisms such as the production of a larger number of sympathetic nerve cells at the expense of germinal or pluripotential cells present in the ganglia. The presence in the ganglia of a fairly

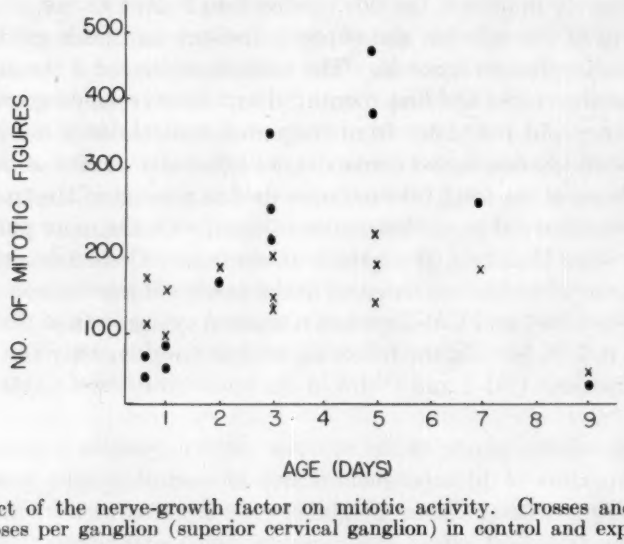

FIG. 1.—Effect of the nerve-growth factor on mitotic activity. Crosses and dots indicate number or mitoses per ganglion (superior cervical ganglion) in control and experimental mice respectively.

large number of small-sized cells, beside the satellite cells, could suggest this possibility. On the other hand, since the mitotic activity is still high at birth and comes to an end at 9 days, the injection of the nerve-growth factor in newborn mice could also affect this process. Counts of mitotic figures in control and experimental ganglia between 1 and 9 days showed a sharp increase in the mitotic activity of the experimental ganglia between 3 and 7 days with a peak at 5 days. These results, presented in Figure 1, favor the hypothesis that the increase in nerve cells in the injected mice is due to an increase in mitotic activity. Although there is no way of deciding how many of the dividing cells are neuroblasts, the results correlate well with the finding of an increase in cell number in later stages; they are also in agreement with observations on the effects of nerve-growth agents on mitotic activity in the spinal ganglia of the chick embryo.[9]

(*c*) *Peripheral distribution of sympathetic nerve fibers:* One would expect that the increase in cell number and cell size in sympathetic ganglia of the injected mice

would result in a parallel increase in the size of nerves emerging from the enlarged ganglia. This increase was in fact observed and it is documented in Figures 10 and 11. A comparison of the cephalic, thoraic, and abdominal regions of experimental and control mice shows an increased density and thickness of the nerve plexuses around the blood vessels of the injected animals. In the kidney, sympathetic nerve bundles were traced along the intrarenal blood vessels and also among the renal tubules, in much larger number in the experimental than in control animals. Figures 14 and 15 show the increase in thickness of the sympathetic nerve along the renal artery in a 19-day injected mouse compared to a control of the same age.

These effects, the increase in number and size of sympathetic nerve cells and the hyperneurotization of the viscera, are very similar to the effects called forth by mouse sarcomas, snake venom, and the salivary extract in the chick embryo.[1, 2, 5, 7] The two sets of experiments differ in two respects: (1) While in chick embryos, the sympathetic nerve fibers produced in excess follow in many instances anomalous routes and even force their way into the lumen of blood vessels, no such deviations were observed in mice injected with the salivary gland fractions. Thus the quantitative rather than the qualitative aspect of the distribution of sympathetic nerves seems to be affected in mammals in post-natal periods. (2) Whereas these agents evoke in the chick embryo a striking response also in the sensory ganglia,[2, 9] this effect is barely noticeable in newborn mice. Measurements of a number of sensory ganglia showed only a slight increase in the experimental material.

TABLE 1

TOTAL NERVE CELL NUMBERS IN SYMPATHETIC GANGLIA OF MICE INJECTED WITH SALIVARY GLAND FRACTIONS

Fraction	Age	Ganglia	Control	Experimental	Ratio Exp./Cont.
CM-3	12	Thoracic N. 6	1,365	3,212	...
		Thoracic N. 7	1,165	2,731	...
		Thoracic N. 8	1,155	4,422	...
		Thoracic N. 9	1,026	2,849	...
		Thoracic N. 10	1,465	2,567	...
		Total	6,176	Total 15,781	2.5
CM-3	19	Stellate			
		Thoracic N. 2	14,400	30,800	...
		Thoracic N. 3	1,644	3,684	...
		Thoracic N. 4	1,770	2,133	...
		Thoracic N. 5	1,094	3,144	...
		Thoracic N. 6	820	2,328	...
		Thoracic N. 7	1,362	2,758	...
		Thoracic N. 8	1,392	4,162	...
		Total	22,482	Total 49,009	2.18
CM-1	Adult	Stellate	14,000	13,689	...
		Stellate	12,000	...	...
		Average	13,000	13,689	1.05
	Adult	Sup. Cerv.	13,300	15,770	...
		Sup. Cerv.	16,415	...	...
		Sup. Cerv.	15,652	...	...
		Average	15,122	15,770	1.03

(*d*) *Effects of fraction CM-1 on adult mice:* The daily injection of 0.05 ml per body weight of fraction CM-1 (6,000 units per ml) was well tolerated and the injected

mice did not show any adverse effects. The sympathetic ganglia were considerably larger than in controls: the difference was evident at a macroscopic inspection of the 50 injected and 50 control mice. Area measurements of the ganglia in a few of cases gave a twofold enlargement, compared with controls. The size increase was due to cellular hypertrophy of the individual neurons (Fig. 9). The cell number was apparently not increased (see Table 1).

Nerve-Growth Effects of Blood Serum of Adult and Young Mice.—Previous experiments *in vitro* gave evidence of a mild nerve-growth effect elicited by embryonic mouse heart on sensory and sympathetic ganglia of chick embryo.[3] Traces of the same activity were then found in homogenates of striated muscle of adult mice. Bueker[10] detected activity in partially purified preparations of thymus, kidney, and muscle. We found in some instances evidence of activity in the urine of adult mice. The same activity was detected in mouse saliva. We then proceeded to test the serum of adult and weanling mice.

Observations to be reported in detail elsewhere disclosed a considerable sexual dimorphism in the sympathetic nerve cells of adult mice. In male mice, the sympathetic neurons are considereably and consistently larger and more intensely stained with basic dyes than in female mice of the same size. These results suggested tests of the blood serum of mice of the two sexes separately. The results are summarized in Table 2. A maximal effect was obtained from the serum of 15 male

TABLE 2

NERVE-GROWTH EFFECTS OF MOUSE SERUM *in vitro*

		Response*			
Age	Concentration	++++	++	+	±
Adult males	1/10	15			
	1	10	30	20	25
Adult females	1	1	7	10	32
Weanling	1		4		26

* ++++ to ± give degrees of effects from maximal to barely detectable.

mice and one female mouse. In 15 cases with a 4+ effect at a dilution of 1 : 10 (Fig. 4) an effect was still detectable at a dilution of 1 : 1000. The nerve-growth effect of the serum collected from adult females or from weanling mice of both sexes was consistently milder than that of the serum of adult males. These sex and age differences have a parallelism in the finding of Cohen (these PROCEEDINGS, this issue) of a higher specific activity in the submaxillary salivary gland of male than of female mice and of much lower activity in the same gland of weanling mice. These results indicate that the nerve-growth factor is present in higher concentration in the blood of adult male than female mice and that it is in even lower concentration in the blood of weanling mice. They also show a considerable variation among mice of the same group. Experiments in progress are expected to answer the question of whether such variations are correlated with physiological differences in the tested animals and if the stress resulting from the administration of the anesthetic may account for such variations.

Effects of the Extirpation of the Submaxillary and Sublingual Glands in Adult and Weanling Mice.—The operated mice were sacrificed between two weeks and six months after the operations and were compared with controls of the same size.

When mice in the weanling stage were operated, the controls were selected from the same litters.

In the first days after the operation, the animals showed signs of discomfort and the fur became ruffled. In the following days, the mice recovered, but in most instances the fur remained deranged. The operated adult males differed from controls also in another respect. They became much more tame than controls and easier to handle. The correlation between submaxillary salivary glands and other endocrine glands, in particular sex glands, have been investigated by many authors[11, 12] and will not be discussed here. They are mentioned, however, since they may have a bearing on the present results.

In all the experimental and control mice, the sympathetic chain ganglia were dissected out, stained with toluidin blue, and sectioned at 10 microns. Since the superior cervical ganglion might have been indirectly affected by the extirpation of part of its peripheral field of innervation, the salivary complex, we used instead the stellate ganglion which does not contribute to the innervation of the salivary glands.

Results.—Twenty operated adult males and twenty controls were examined between three weeks and two months after the operation. In five mice deprived of the salivary glands a slight decrease in size of the sympathetic nerve cells was detected. The nerve cells also stained less intensely than controls with toluidin blue and appeared similar to sympathetic nerve cells of female mice. No differences were noticeable in the other 15 operated mice. Five adult female mice were operated and compared with controls three weeks after the operation. No size differences were detected between these and control nerve cells. Equally negative were the results in 5 mice operated during the weanling stage. One of these was compared with a control of the same litter, six months after the operation, the other mice one month after the operation. In all instances, the sympathetic chain ganglia appeared of the same size as the controls and the histological examination of the stellate ganglia revealed no changes in cell size.

The serum of mice deprived of the salivary glands was also tested for the nerve-growth factor. In one mouse deprived of the glands two months earlier the serum evoked a 4+ effect *in vitro* as the serum of control mice. In the other specimen the effect varied from a 2+ to a barely detectable effect. These results indicate that the nerve-growth agent is present, even in the absence of the salivary glands.

Summary.—Previous work has provided evidence for the presence of nerve-growth promoting agents in a variety of biological materials: mouse sarcomas, snake venoms, and mouse submaxillary salivary glands. In the present investigation the effect of the active fraction isolated from the mouse salivary glands was tested *in vitro* on ganglia of human fetuses; it was found that it elicits the same effects as on ganglia of other species. The active fraction was then injected in newborn and in adult mice. In all instances the injection resulted in a marked increase of the sympathetic ganglia; the response varied with the age of the animal, the amount, and the purity of the fraction injected. In some instances a sixfold increase in size was observed. Slight nerve-growth promoting effects of different mouse tissues had been observed *in vitro* in previous experiments. We have now found evidence for the presence of the nerve-growth factor in the serum of adult and weanling mice. Maximal effects were obtained from the serum of adult male mice; the serum of female mice is less effective. This sex difference is paralleled by a sex difference in the size of adult sympathetic nerve cells.

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† We wish to express our appreciation to Dr. Willard Allen of the Department of Gynecology and Obstetrics of the Washington School of Medicine who made this material available to us.

‡ The microphotographs in this and in the following paper were made by Mr. Cramer Lewis of the Department of Illustration of the Washington University School of Medicine.

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*DESTRUCTION OF THE SYMPATHETIC GANGLIA IN MAMMALS BY AN ANTISERUM TO A NERVE-GROWTH PROTEIN**

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The observation by S. Cohen that a rabbit antiserum against a protein fraction of the salivary gland inactivates the *in vitro* nerve-growth effects of this protein (pp. 302–311, these PROCEEDINGS) suggested to him to test the effects of the antiserum on newborn mice. The finding of a remarkable decrease in size of the sympathetic ganglia of the injected mice prompted an extensive investigation of the effects of the antiserum on the sympathetic ganglia of mice and other mammals. The results of this study are reported in the following pages.

Materials and Methods.—Newborn mice were injected daily with 0.05 ml of the rabbit antiserum per 1.5 gm of body weight.† Controls of the same litters were either injected with serum of a normal rabbit, or they were not treated at all. Twenty experimental and twenty control mice were sacrificed between the 12th hour after the first injection and the 25th day. Two groups of newborn mice, injected for 8 and 20 days respectively after birth, were sacrificed three and four months after the termination of the treatment. An equal number of untreated mice of the same litters were available for control. The same techniques as used in the previous experiments (pages 373–384) were used for area measurements, cell and mitotic counts, and for histological examination.

The effect of the rabbit antiserum was then tested on newborn rats, rabbits, and one pair of kittens. The amount injected was in the same proportion to the body

weight as in the experiments with newborn mice. A limited number of experiments were also performed on adult mice and on one pair of young adult squirrel monkeys.

Effects of the Antiserum on Newborn Mice.—The injection of the rabbit antiserum in newborn mice did not affect their development in any way. Mice injected daily for a period of 25 days did not differ from mice injected with normal rabbit antiserum or from untreated controls. Since mice injected with the normal serum were identical in all respects with untreated controls, including the development of the sympathetic ganglia, only untreated animals were used for comparison in most experiments.

The effects of the antiserum on the development and growth of the sympathetic ganglia were dramatic in their sudden outburst, in their severity, and in the end effects. An inspection of sympathetic ganglia at different trunk and head regions in the same animals showed that all ganglia were affected in the same way; therefore, the data presented here on the superior cervical ganglia can be considered as representative of the degree of reduction which took place in all sympathetic ganglia.

A decrease in mitotic figures is already noticeable 12 hours after the first injection; the decrease becomes more pronounced at the end of the first day. Between the second and the third day, the mitotic activity is sharply reduced and the number of degenerating cells is greatly increased. The neuroblasts which are present are considerably smaller than the control cells. As a result, the volume of the ganglia is reduced to approximately $^1/_6$ of the controls on the fourth day. No cell counts were performed at this stage since most of the neuroblasts are still small and not easily distinguishable from satellite cells.

Between the fourth and the ninth day, the process of cell atrophy and death continues at a somewhat slower pace; the disintegrative processes of preceding stages have in fact so much reduced the population of nerve cells as to set limits to the process itself.

The effects of the daily injections of antiserum in a 9-day mouse are illustrated in Figure 5. The products of disintegrating cells which in earlier stages encumbered the ganglia have now disappeared. A few nerve cells, smaller than controls but otherwise in apparently normal condition, are scattered among a uniform population of satellite cells. The latter do not seem to be affected by the antiserum at this stage. A reduction in their number takes place in more advanced stages when only a few satellites are still present in each section of the diminutive ganglia (Fig. 8).

At 20 days, counts of nerve cells in the superior cervical ganglia of experimental and control mice show that the sympathetic nerve cells are reduced in one instance to less than 1 per cent of the control (Fig. 1, Table 1). The reduction of volume of the same ganglion was to 4.3 per cent of the control. The process seems to have reached its peak. Counts of cells in another pair of experimental and control ganglia of a 25-day-old mouse give slightly higher figures: 1.7 per cent of the cell population of the control are present in the experimental ganglion. Such differences may well be accounted for as individual variations. It should be noted that a considerable variation in the cell population was also observed in ganglia of untreated mice (see Table 1). The results, seem, however, to indicate that the process has not

further advanced, and that the few cells which escaped death in earlier stages may now be able to survive.

It was of interest, in this connection, to examine the injected mice some months after the termination of the treatment. Seven newborn mice of two litters were injected daily with the antiserum until the eighth and the twentieth day respectively. Experimental and untreated mice of the same litters were examined three to four months later. The treated mice did not differ from controls in their general appearance. The lack of adverse effects on the visceral functions of the injected mice was illustrated by the fact that one of these mice delivered a normal litter.

Two experimental and two control mice of each group were sacrificed at the end of the third month and the sympathetic chain ganglia were dissected out and compared. Two more pairs of mice injected for 8 days were sacrificed at the end of the fourth month (Figs. 2, 8). The results are given in Table 1. The reduction in cell number in the superior cervical ganglia fluctuates between 2.56 per cent and 0.84

TABLE 1

EFFECT OF ANTISERUM INJECTIONS ON THE VOLUME AND CELL NUMBER OF THE SYMPATHETIC GANGLIA IN MAMMALS*

Mammal	Age, Days	No. of Inject.†	Ganglion	Volume Ratio, Exp./Cont.	Number of Cells Cont.	Number of Cells Exp.	Cell Ratio, Exp./Cont.
Mouse	2	2	Sup. Cerv.	0.37:1			
Mouse	4	4	Sup. Cerv.	0.16:1			
Mouse	9	9	Stellate	0.13:1			
Mouse	20	20	Sup. Cerv.	0.04:1	13,300	91	0.70:100
Mouse	25	25	Sup. Cerv.	..	16,415	279	1.70:100
Mouse	90	8	Sup. Cerv.	..	16,447	421	2.56:100
Mouse	120	8	Sup. Cerv.	..	14,800	140	0.94:100
Mouse	120	20	Sup. Cerv.	..	13,000	110	0.84:100
Mouse	Adult	20	Sup. Cerv.	..	15,652	5,328	34.00:100
Rat	4	4	Sup. Cerv.	6.64			
Rat	7	7	Sup. Cerv.	10.54	32,000	2,310	7.00:100
Rabbit	$3^1/_2$	3	Sup. Cerv.		66,300	6,200	9.00:100
Rabbit	5	5	Sup. Cerv.		67,000	9,300	14.00:100
Rabbit	7	7	Sup. Cerv.		68,000	11,050	16,00:100
Cat	7	7	Sup. Cerv.		114,000	8,600	7.70:100
Monkey	7	7	Sup. Cerv.	1.65			

* Details of experiments in text.
† Beginning at birth, except for adult mice.

per cent of the corresponding control ganglia. In all instances, the residual cells were much smaller and did not stain with basic dyes as deeply as the controls. The function of the surviving cells will be tested in other mice of the same litters which are still alive. The 20-day treatment, as well as the 8-day treatment, is therefore sufficient to destroy 97 per cent to 99 per cent of the sympathetic nerve cells. Since the mice were examined some months after the end of the treatment, it is proved that the damage inflicted to nerve cells is irreversible. Experiments with higher amounts of antiserum and possibly an even more potent antiserum will show whether it may be possible to attain a total extermination of the sympathetic nerve cells.

Effects of the Antiserum in Other Newborn Mammals.—The few experiments performed up to now on newborn rats, rabbits, and kittens are not sufficient to give more than a very incomplete idea of the effects of the antiserum in these species.

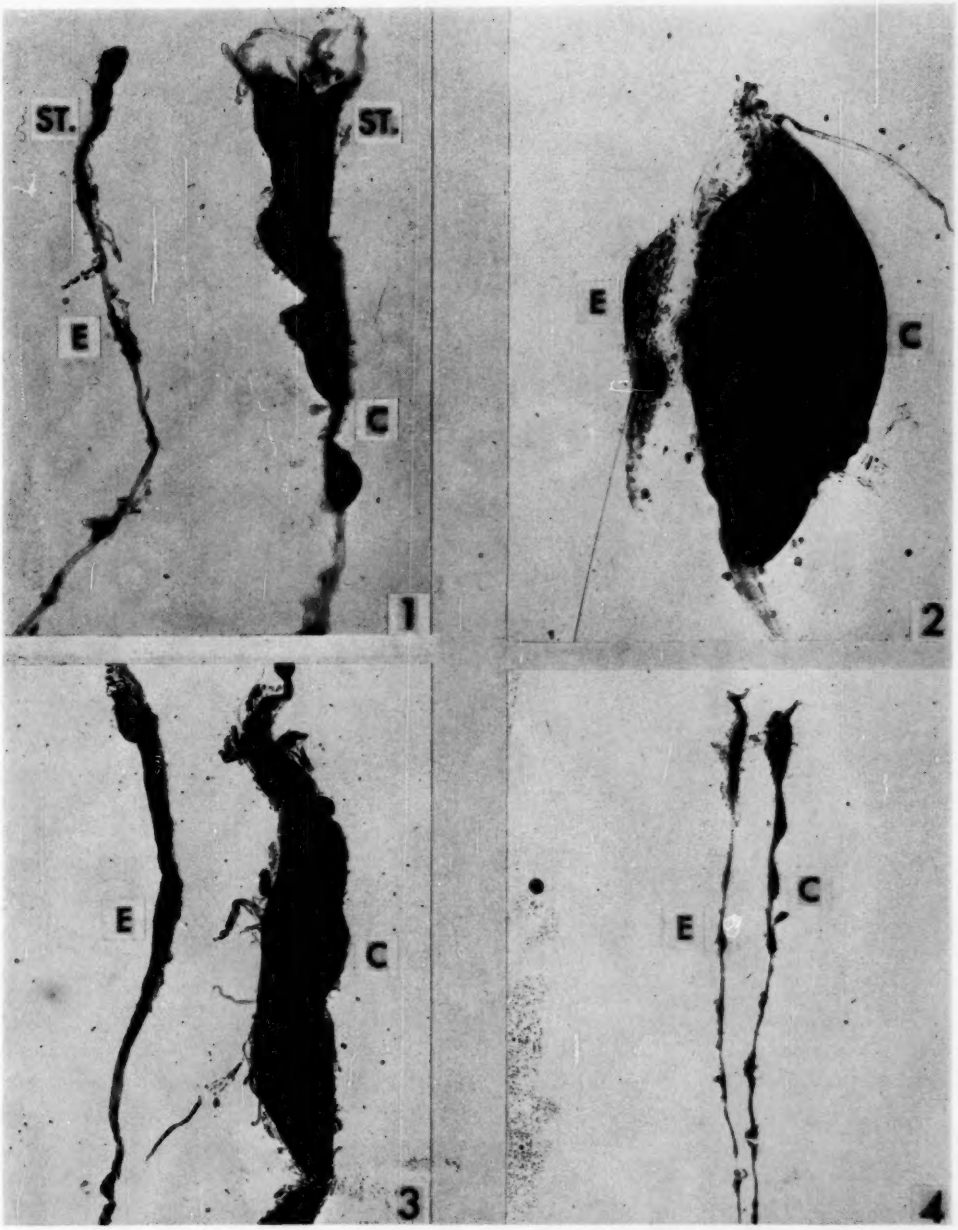

PLATE I.—Effects of rabbit antiserum against the purified protein of mouse salivary gland.

FIG. 1.—Stellate (St.) and first ganglia of sympathetic thoracic chains in control (C) and experimental (E) 20-day old mice. Experimental mouse injected daily since birth with the antiserum to the mouse salivary protein. 30×. FIG. 2.—Superior cervical ganglia in control (C) and experimental (E) 4-month old mice. Experimental mouse injected daily from birth to the 8th day with the antiserum to the salivary protein. 33×. FIG. 3, 4.—Effects of the antiserum in a 7-day old rat (FIG. 3) and in a 3½-day old rabbit (FIG. 4); both injected daily since birth. FIG. 3, stellate ganglia in control (C) and experimental rat (E). 30×. FIG. 4, stellate and sympathetic thoracic chains in control (C) and experimental (E) rabbit. 4×.

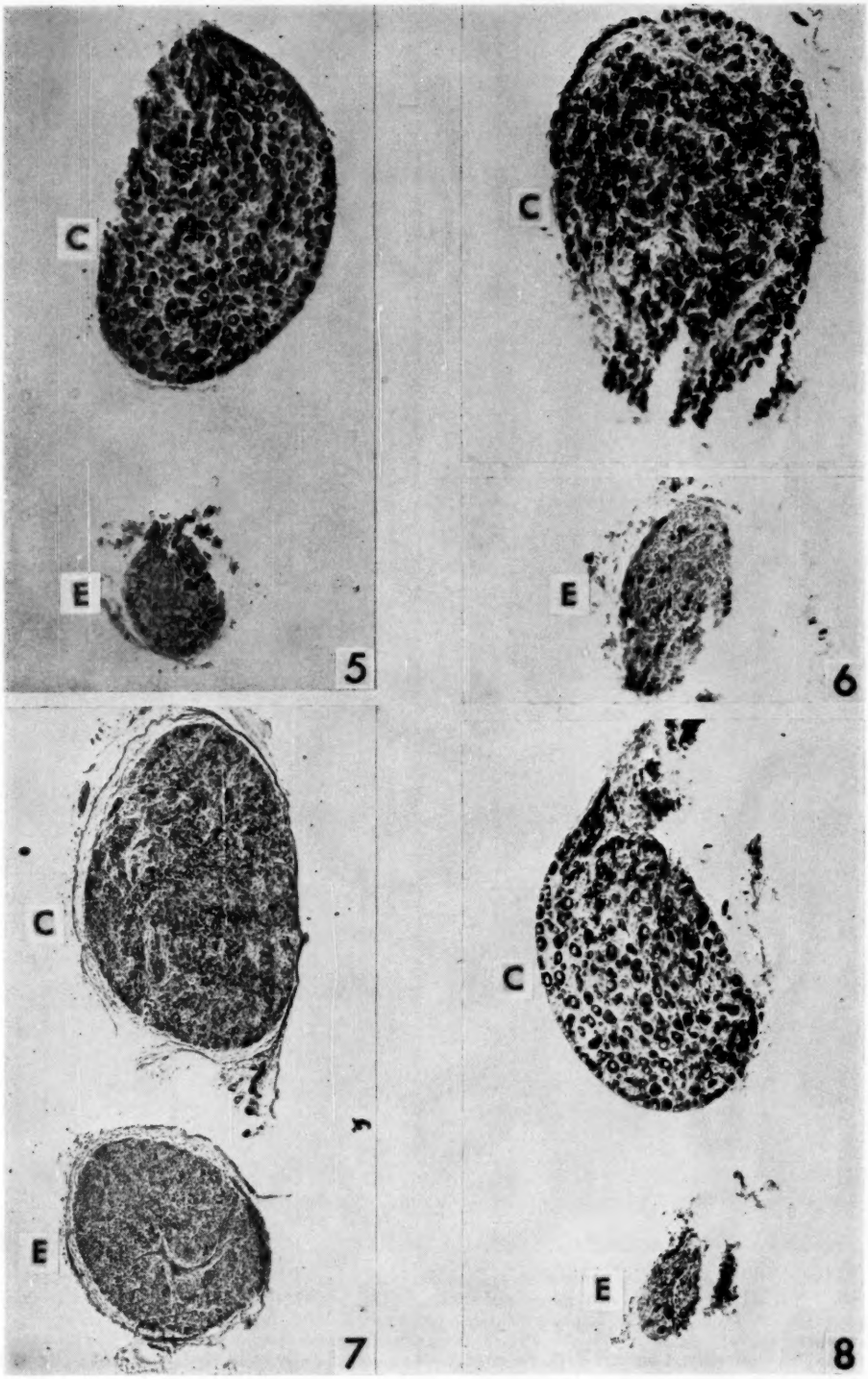

PLATE II.—Transverse sections of superior cervical ganglia in control (C) and experimental (E) animals, injected with rabbit antiserum to the salivary protein.

FIG. 5.—9-day old mice; E, injected daily since birth. 90 ×. FIG. 6.—7-day old rats; E, injected daily since birth. 90×. FIG. 7.—7-day old kittens; E, injected daily since birth; the ganglion consists of satellite cells and few nerve cells. 30 ×. FIG. 8.—4-month old mice; E, injected from birth to the 8th day. 60×.

The results obtained are, however, so impressive as to justify the contention that the antiserum affects the sympathetic ganglia of these mammals in a similar way as described above for newborn mice (Figs. 3, 4, 6, 7).

Two newborn rats were injected with the antiserum (0.05 ml per 1.5 gm of body weight). The experimental and control animals were sacrificed respectively at 5 and 7 days. The results observed are so similar to the ones described in mice at the same developmental stages as to make a description unnecessary. The number of cells in the superior cervical ganglion of one injected rat at 7 days was reduced to 7 per cent of the control (Table 1). The surviving cells are reduced in size when compared to controls but otherwise apparently in good condition (Figs. 3, 6).

Three newborn rabbits were injected with the rabbit antiserum and sacrificed at $3^1/_2$, 5, and 7 days. Since the ganglia are rather large in this species and the nerve cells easily distinguishable from satellites even in earlier stages, cell counts were feasible in three-and-a-half day old ganglia. A high reduction in the number of nerve cells was observed: only 9 per cent of the control population is present in the injected rabbit at this stage (Fig. 4). The two rabbits of the same litter, injected and sacrificed two and four days later respectively, showed a somewhat less severe atrophy of 14 per cent and 16 per cent respectively. Furthermore, the surviving cells were not reduced in size as in the younger animal. Investigation of animals injected for a longer period will answer the question whether rabbits develop a resistance to the antiserum which could account for such results.

The effects of the antiserum in one adult mouse, one young squirrel monkey, and a newborn kitten are shown in Table 1.

Discussion.—The results reported in this and in the previous paper bring to light the remarkable capacity of sympathetic nerve cells of newly born mammals to increase in size and number when injected with a mouse salivary gland protein, as well as their vulnerability to an antiserum to this protein.

The possibility that the sympathetic ganglia in man may be likewise affected by the nerve-growth factor is suggested by the *in vitro* experiments reported in the preceding paper.

The salient aspects of these phenomena are: their universality, their target specificity, and the unsuspected growth potentialities of young and adult nerve cells which materialize under the impact of nerve-growth promoting agents.

Of particular significance is the new finding that the antiserum against the salivary gland protein not only inactivates this protein, but, if injected alone into a newborn mammal, destroys its sympathetic cells almost totally in a remarkably short time. No other organs and tissues appear to be affected. On the other hand, normal serum leaves the sympathetic system as well as all other systems intact.

The question arises whether the near-total destruction of the sympathetic nerve cells is a direct or an indirect effect of the antiserum. It is conceivable that in the normal animal a factor circulates which is necessary for growth and maintenance of the sympathetic system and that the antiserum inactivates this factor. This hypothesis is supported by the detection of the nerve-growth agent in the blood of young and adult mice. Alternatively, the antiserum may exert a cytotoxic affect by combining with the antigen in or on the surface of the nerve cells. The same questions are raised in other investigations of the cytotoxic effects of the antisera.[1] Immuno-

chemical techniques, such as the detection of antibodies labeled with a radioactive isotope or with a fluorescent dye, may show if there is localization of the antiserum on the sympathetic nerve cells and if so, provide evidence in favor of the second alternative. These experiments will be performed in the near future.

The results reported in these and in previous papers[2-5] give evidence for the existence of closely related nerve-growth promoting proteins in two seemingly unrelated sources: mouse sarcomas and salivary glands. One may wonder whether both structures produce these proteins. We have positive evidence that the mouse sarcomas manufacture the nerve-growth agent even when they grow in the chick embryo.[6,7] Corresponding experiments in which the salivary glands were transplanted in the chick embryo were negative so far. Furthermore the nerve-growth agent was detected in serum of adult mice which had been deprived of their salivary glands two months earlier. The sympathetic ganglia of these mice did not show appreciable differences from control mice. Since the salivary glands and their homologue, the snake venom glands, contain the agent in high concentration, the only alternative would be that these glands store, but do not produce, the growth agent. If this suggestion would prove to be correct, we would still be faced with the problem of identifying the source of the nerve-growth agent in the organism. The possibility ought to be considered that the nerve-growth agent is not manufactured in any particular organ or organs, but in the mesenchymal tissue. The only evidence in favor of this hypothesis comes from the *in vivo* and *in vitro* experiments with mouse sarcomas and from *in vitro* experiments with explants of embryonic mouse heart.[8] Epithelial structures like carcinomas proved to be completely deprived of the property of stimulating nerve growth *in vivo*[9] as well as *in vitro*.[8] Research in progress is now aimed at the investigation of this problem.

Finally, we should like to call attention to other aspects of these experiments. They gave evidence of the high tolerance of the organism for such deviations from normality as a sixfold increase in volume of the sympathetic ganglia, or their near-total extinction. The injected animals apparently did not differ from controls when raised and observed in the sheltered conditions of the laboratory. Physiological tests are expected to reveal these differences and will therefore be applied to the treated and control animals.

Summary.—We have reported the effects of an antiserum to the nerve-growth factor which selectively destroys most of the sympathetic nerve cells in a variety of newborn mammals. Daily injections for a period of 8 days resulted in the disappearance of up to 99 per cent of the sympathetic nerve cells. This process is irreversible. It is suggested that the salivary glands do not produce the nerve-growth agent but merely accumulate and store it. The mode of action of the antiserum and the possible role of the nerve-growth agent as a maintenance factor for sympathetic neurons are under investigation.

* This work has been supported by a grant from the National Science Foundation and by a contribution from an institutional grant of the American Cancer Society to Washington University.

† For preparation of the antiserum see paper by Cohen, S., 302–311, these PROCEEDINGS.

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